ACTA ALIMENTARIA

ACADEMIAE SCIENTIARUM HUNGARICAE

EDITED BY

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VOL. 4 NUMBER 1

AKADÉMIAI KIADÓ, BUDAPEST

1975

ACTA ALIMENTARIA

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A QUARTERLY OF THE COMMITTEE ON FOOD SCIENCE
OF THE HUNGARIAN ACADEMY OF SCIENCES

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Address of the Editorial Office:

Central Food Research Institute
H-1022 Budapest, Herman Ottó út 15. Hungary

Acta Alimentaria is a quarterly publishing original papers on food science in English. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Acta Alimentaria is published in quarterly issues comprising about 400 pages per year.

Subscription price: $32.00 per volume

Distributor:

KULTURA

Hungarian Trading Co. for Books and Newspapers
Budapest 62, P.O. Box 149, Hungary
or its representatives abroad, listed on p. 3 of the cover.

Acta Alimentaria is published by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
Budapest 502, P.O. Box 24, Hungary

Acta Alimentaria, Vol. 4. (1), pp. 1—21 (1975)

OBJECTIVE DETERMINATION OF THE HEAT TREATMENT REQUIREMENT OF COOKED POTATOES

E. Kovács, K. Vas and J. Bencze-Bőcs

(Received March 16, 1973)

Hardness of 5 potato varieties (Gülbaba, Bintje, Désirée, Maryke, Jaerla) was studied during cooking at various temperatures (75 – 95 °C), as a function of cooking time (0 – 20 min) and of time of previous cold storage (0 – 6 months at 5 – 10 °C). Hardness was measured instrumentally (*Texturometer*) and evaluated sensorically.

It was found that cooking times, necessary to achieve 50% reduction in hardness, were similar for all varieties. Cooking times, needed to decrease hardness to a definite absolute value (*e.g.*, *Texturometer* values 45 or 30), however, varied slightly with the variety, the slowest-cooking varieties being Bintje and Désirée.

Appreciable differences in instrumentally measured cooking velocity could be demonstrated between potatoes before and after 5 or 6 months of cold storage. The potato lots stored for longer periods showed quicker softening under the same cooking conditions.

Cooking velocity showed linear increase with increase in temperature. No varietal effect could be observed. Q_{10} values ranged between 1.8 and 2.9, averaging around 2.0, and exhibited no dependence on variety or storage time.

Sensory and instrumental methods for determining hardness in potatoes showed some correlation. The instrumental measurement proved to be more sensitive and applicable to a wider range of hardness values than did sensory evaluation.

In the knowledge *(a)* of the temperature history of the heat-treated tubers, *(b)* of the temperature dependence of cooking velocity and *(c)* of the objective (mechanical) standard of the hardness characteristics of the cooked state, the "cooking requirement" can be calculated. In this way the exact requirements of heat treatment can be determined and degradation of texture and wastage of heating energy, both being consequences of overcooking, can be avoided.

An ever-growing portion of the potato crop is being processed by the food industry (Hampson, 1969; Paulus, 1971; Uhlmann, 1972). One of the processing methods is heat treatment. Of the various forms of heat treatment, cooking is an important operation not only from the point of view of culinary technology, but also from that of the production of canned (sterilized) potatoes which appear to be in great demand with the consumers in many countries.

Apart from microbiological considerations, knowledge of the heat treatment required to bring about the "cooked" state of the potato, so as to avoid unnecessary overcooking (*i.e.*, undesirable texture, energy wastage, *etc.*), is of technological and economic importance.

Exact determination of the cooking requirement is fairly difficult at present. However, it was thought possible to solve this problem *(a)* by finding an objective standard for the cooked state, *(b)* by determining the rate

of cooking *(i.e.*, of "getting cooked") as a function of temperature, and *(c)* by recording the temperature history of potato tubers under the actual cooking operation. It was assumed that in this way, the heat treatment requirement ("cooking requirement") could be calculated for every process.

1. Materials and methods

1.1. Potatoes used in the experiment

The following varieties were tested.

Gülbaba. Hungarian variety (developed by J. ESZENYI and V. TEICHMANN, 1940). The tuber is long, cylindrical, of uniform surface. Shallow-seated eyes are situated on the tip of the tuber, sometimes the eyes protrude. The skin is pale red and smooth. The flesh is white, the glomerules show up hazily.

Quality characteristics: Early maturing, medium yield table potato variety with excellent eating quality. Starch content: 15—16% (O.M.F.I., 1970).

Bintje. Tuber: long oval, flattened, larger-sized; peel: ochre-coloured; the flesh is light yellow. The eyes are shallow-seated.

Quality characteristics: Table potato of medium earliness with a high yield and low starch content. Excellent eating quality, does not disintegrate on cooking, only slightly mealy. Does not show grey darkening upon cutting and cooking. Good storage quality.

Jaerla. Tuber: roundish oval-shaped; peel: smooth, shiny and yellow; flesh: light yellow, eyes: fairly shallow-seated.

Quality characteristics: Very early growing table potato with low starch content, non-mealy, does not disintegrate on cooking. Good eating quality. Moderate storage life.

Maryke. Tuber: large to very large, long oval shape, eyes seated on the surface. Peel and flesh: light yellow.

Quality characteristics: Moderately late-growing table potato with high starch content; mealy. Good eating and storage qualities.

Désirée. Tuber: large, long oval, regular-shaped with red skin and light yellow flesh. Eyes seated near the surface.

Quality characteristics: Moderately late-growing potato variety with low starch content, very slightly mealy. Moderate storage quality (O.M.F.I., 1973).

Potato consignments were bought from the *Co-operative Enterprise for Agricultural Products* (Miskolc) in October 1972, except for Gülbaba which was bought in May 1973 from the *Agricultural Co-operative Sasad* (Budapest).

Storage of the potatoes was carried out at a temperature of 5—10 °C at relative humidities between 60 and 70%.

1.2. Cooking experiments

1.2.1. Preparation of raw material. Peeled potatoes were used in the experiments in two forms, *(a)* cut across the long axis of the tuber into discs of 15 mm thickness, with a diametre of 30—35 mm except for the variety Maryke which had an average diametre of 70—80 mm, and *(b)* cut into discs of 1.5 $\pm$ 0.2 mm, with a diametre of 26 mm. This diametre was secured by first producing potato cylindres by the use of a cork-borer of 26 mm diametre and then cutting these cylindres with a cucumber slicer.

1.2.2. Heat treatment. In all cases, a 1.5% NaCl brine was used as the heat transfer medium. The ratio of brine to potato was 2 to 1 in the case of the 15-mm discs and 10 to 1 with discs of 1.5 mm thickness. In this way, it could be achieved that, on introducing the potato slices into the brine of predetermined temperature, the drop in the latter was very low (with 15-mm discs: 0.5—1.0 °C for less than 3 min in a brine of 95 °C) or nil (with 1.5-mm slices).

Cooking temperatures applied were as follows: 75, 80, 85, 90 and 95 °C.

Cooling to room temperature was done by decanting the brine.

1.3. Measurement of consistency

Mechanical properties of potatoes were measured at room temperature with a *Texturometer* manufactured by the *Zenken Co.*, Japan, under licence from *General Foods Corp.*, USA. The conditions of measurement were as follows:

— dentures bronze plunger,
— articulator speed: 12 chews per minute,
— recording paper speed: 750 mm min^{-1},
— receiver for the electric signal operative in the range of 0—2.5 V (at 0.5 V),
— "Attenuator 1" adjustment.

Hardness of the 1.5-mm slices was measured by piling 4—5 discs on each other to reach an integrated thickness of 6.7—7.0 mm.

Evaluation of texture measurements was carried out as described by KOVÁCS and VAS (1969). The height of the peak was taken as the measure for hardness.

1.4. Sensory tests

To determine the "cooked state" of potatoes and to correlate sensory with instrumental evaluation, 15-mm discs were cooked at 95°C for varying periods, and tested by 5—10 tasters with the same lots of potatoes on two

occasions (November 1972 and May 1973). The scoring system used was as follows:

5: very hard,
4: slightly hard,
3: optimal texture,
2: slightly soft,
1: very soft.

2. Results

Changes in the texture of a number of potato varieties were measured as a function of cooking temperature and time by using an instrumental method and by sensory testing.

2.1. Changes in hardness during cooking at 95 °C

15-mm discs of potatoes were cooked at 95 °C for 5, 10, 15 and 20 min, resp., and their hardness determined, after quick cooling to room temperature, with a *Texturometer*.

Measurements in November were repeated on the same lots of potatoes after about 6 months of storage at 5—10 °C in May.

Table 1 and Fig. 1 show the changes in hardness of 5 potato varieties as a function of cooking time at 95 °C.

As can be seen, average hardness values range from 345.0 to 506.6 for the 5 potato varieties in the raw (uncooked) state. Analyses of variance showed that there was a highly significant difference in hardness between the varieties tested in November, and a very highly significant difference between varieties tested in May. The differences in hardness between varieties did not disappear after cooking for 5, 10 and 15 min. By analysis of variance, all these differences were found to be very highly significant statistically.

In the course of cooking, the greatest changes occur in the first 5 (or 10) minutes. Changes during this period also determine the difference in "cookability" of the same variety before and after storage for 6 months. It is evident that potatoes, stored for a longer period could be softened much quicker by cooking.

The duration of cooking at 95 °C, necessary to reduce hardness values to 50 %, 10 %, 5 % and 2.5 %, resp., of the hardness of the raw potatoes ($t_{50\%}$, $t_{10\%}$, $t_{5\%}$, $t_{2\cdot5\%}$) has been interpolated from a number (N) of individual cooking curves and their means are shown, together with their standard deviations, in Table 2a.

The differences in mean cooking time requirements can be seen in Table 2b.

It is evident, that although, except for Bintje, cooking time requirements of the 5 varieties did not vary appreciably, they were reduced to 40—50% of the original value by storage of about 6 months, although the hardness value in the raw state did not show nearly similar decreases during storage.

Based on Fig. 1, the times required to reach certain predetermined hardness values (*i.e.*, *Texturometer* readings: 45, 30, 20, 15 and 12.5, resp.) during cooking at 95 °C are shown in Table 3.

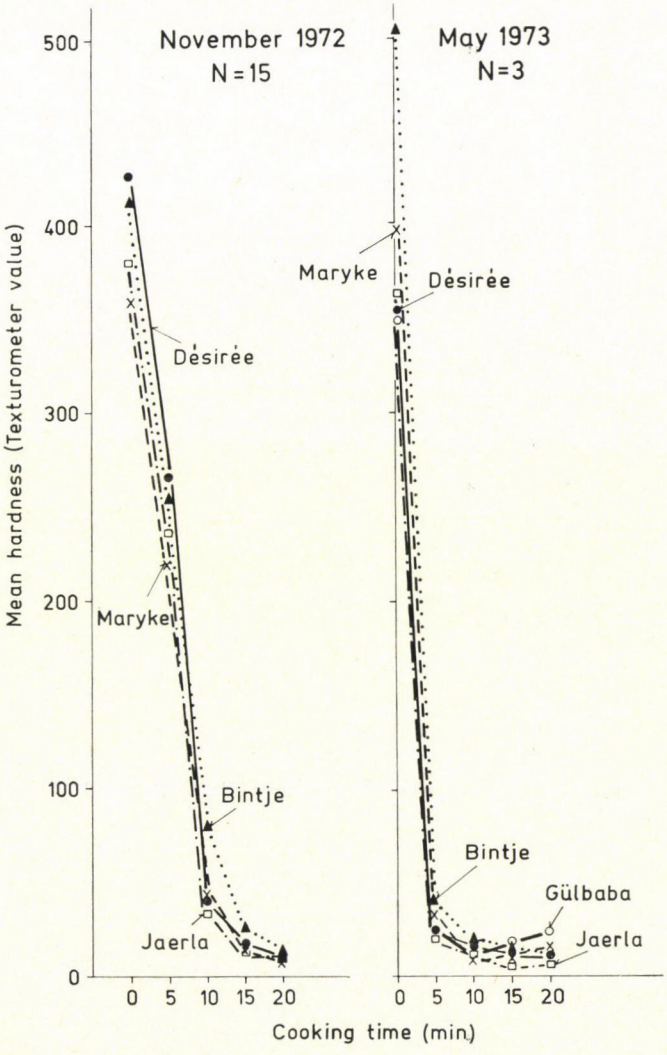

Fig. 1. Changes in the mean hardness values of potatoes as a function of cooking time at 95 °C, before and after cold storage (15 mm thick slices)

Table 1

Changes in consistency of various potato varieties as a function of cooking time, before and after 6 months of storage (15 mm thick slices; 95 °C)

Storage time (months)	Cooking time (min)	N	Variety									
			Désirée		Maryke		Jaerla		Bintje		Gülbaba	
			Texturometer hardness value									
			$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
	0	15	427.1	62.64	358.8	34.76	378.9	63.00	415.0	80.12		
0	5	15	266.3	81.37	217.7	80.23	237.1	86.39	254.5	73.04		
(November 1972)	10	15	40.0	17.06	41.3	24.88	32.9	18.89	81.3	73.68		
	15	15	18.9	8.30	14.9	7.99	13.1	4.44	25.9	19.48		
	20	15	10.8	6.90	9.3	3.51	12.0	11.56	13.9	3.77		
	0	3	355.0	54.91	397.5	41.93	363.7	75.64	506.6	23.63	345.0	19.04
6	5	3	25.0	13.23	33.0	34.91	19.8	20.69	42.3	21.82	33.3	4.16
(May 1973)	10	3	14.7	7.93	8.8	2.75	9.6	3.94	19.8	6.24	12.5	2.18
	15	3	14.6	0.58	7.8	2.25	6.0	2.16	13.0	1.99	19.1	3.29
	20	3	10.3	2.99	11.5	2.99	6.3	1.19	11.9	5.62	23.6	2.93

N: No. of parallels
$\bar{x}$: average hardness value
s: standard deviation

Table 2a

Cooking times necessary, at 95 °C, to reduce hardness to 50, 10, 5 and 2.5% of the original value as measured in raw potato slices (15 mm thickness) before and after 6 months of storage

Storage time (months)	Variety	Hardness of potatoes (*Texturometer* value)	Cooking time requirement (min)											
			$t_{50\,\%}$			$t_{10\,\%}$			$t_{5\,\%}$			$t_{2,5\,\%}$		
			N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s
0	Désirée	427.1	10	*6.0*	1.49	10	*10.3*	1.16	10	*13.6*	1.95	—	—	—
(November 1972)	Maryke	358.8	15	*5.9*	1.43	15	*11.1*	2.00	13	*12.8*	2.39	10	*16.3*	2.23
	Jaerla	378.9	14	*6.0*	1.48	14	*10.1*	1.86	14	*12.6*	2.21	10	*16.6*	2.31
	Bintje	415.0	11	*6.5*	1.20	11	*13.3*	2.76	10	*15.5*	3.54	4	*13.8*	2.17
	Gülbaba	—	—	—	—	—	—	—	—	—	—	—	—	—
6	Désirée	355.0	3	*2.5*	0	3	*5.0*	0.71	3	*9.5*	4.14	1	*8.0*	—
(May 1973)	Maryke	397.5	4	*2.6*	0.27	4	*5.3*	0.99	4	*6.5*	1.66	4	*7.9*	2.19
	Jaerla	363.7	4	*2.5*	0	4	*5.1*	0.84	4	*6.1*	1.67	2	*11.3*	0.25
	Bintje	506.0	3	*2.7*	0.24	3	*5.3*	0.85	3	*7.0*	1.47	2	*15.0*	0
	Gülbaba	345.0	3	*2.5*	0	3	*5.2*	0.24	3	*9.2*	0.62	—	—	—

N = number of parallel measurements
$\bar{x}$ = average
s = standard deviation

Table 2b

Significance of differences in mean cooking time requirements of potato varieties (15 mm thick slices; 95 °C) before and after 6 months of storage (means as recorded in Table 2a)

Storage time (months)	Variety	$t_{50\%}$				$t_{10\%}$				$t_{5\%}$			
		Variety											
		Désirée	Maryke	Jaerla	Bintje	Désirée	Maryke	Jaerla	Bintje	Désirée	Maryke	Jaerla	Bintje
0	Maryke	—				—				—			
(November 1972)	Jaerla	—	—			—	—			—	—		
	Bintje	—	—	—		***	***	***		—	**	**	
6	Maryke	—				—	—			—			
(May 1973)	Jaerla	—	—			—	—			—	—		
	Bintje	—	—	—		—	—	—		—	—	—	
	Gülbaba	—	—	—	—	—	—	—	—	—	**	**	—

— = no significant difference ($P < 95\%$)
** = highly significant difference ($P \geq 99\%$)
*** = very highly significant difference ($P \geq 99.9\%$)

Table 3

Cooking times needed to reduce hardness of raw potatoes. Texturometer values 45, 30, 20, 15, and 12.5, respectively (15 mm thick slices; 95 °C)

Storage time (months)	Variety	Cooking time requirement (min)				
		t_{45}	t_{30}	t_{20}	t_{15}	$t_{12,5}$
0	Désirée	9.70	12.40	14.80	17.60	19.00
(November)	Maryke	9.60	11.80	13.80	15.00	19.00
	Jaerla	9.50	10.80	13.20	14.60	16.40
	Bintje	12.80	14.60	17.40	19.50	15.00
	Gülbaba	—	—	—	—	—
6	Désirée	4.50	6.75	8.75	10.00	17.75
(May)	Maryke	4.00	5.50	7.50	8.50	9.00
	Jaerla	3.25	4.25	5.00	7.50	9.00
	Bintje	4.75	7.75	10.00	13.50	20.00
	Gülbaba	4.25	5.50	8.00	9.50	—

It can be seen that, here, cooking requirements varied depending on the variety, as well as on the length of storage of the potato. The slowest-cooking varieties were Bintje and Désirée.

2.2. Temperature dependence of cooking velocity

Cooking experiments, in a 1.5% NaCl brine having a volume about 10 times that of the potatoes, were conducted with potato discs of 1.5 mm thickness (diametre: 26 mm) at various temperatures (see: para. 1.2.) in December and May, except for variety Gülbaba which was examined in May only. Table 4 and Figs. 2 and 3 show the hardness values of potato varieties as a function of cooking time and temperature.

Cooking times necessary to reduce hardness to 50% and 25% of its original value, respectively, ($t_{50\%}$, $t_{25\%}$) were interpolated from curves in Figs. 2 and 3 and are shown in Table 5.

The reciprocals of $t_{50\%}$ and $t_{25\%}$ were used to express cooking rate or softening velocity ($v_{50\%}$, $v_{25\%}$). These are also given in Table 5.

It is apparent that, with the exception of the variety Gülbaba which was tested after a much longer storage period, the varieties showed no substantial difference in cooking rate at the same temperature levels. At different temperatures, however, cooking times varied quite considerably.

Times required to produce a 50% drop in hardness and to reach a hardness value of 30, respectively, are recorded in Table 6.

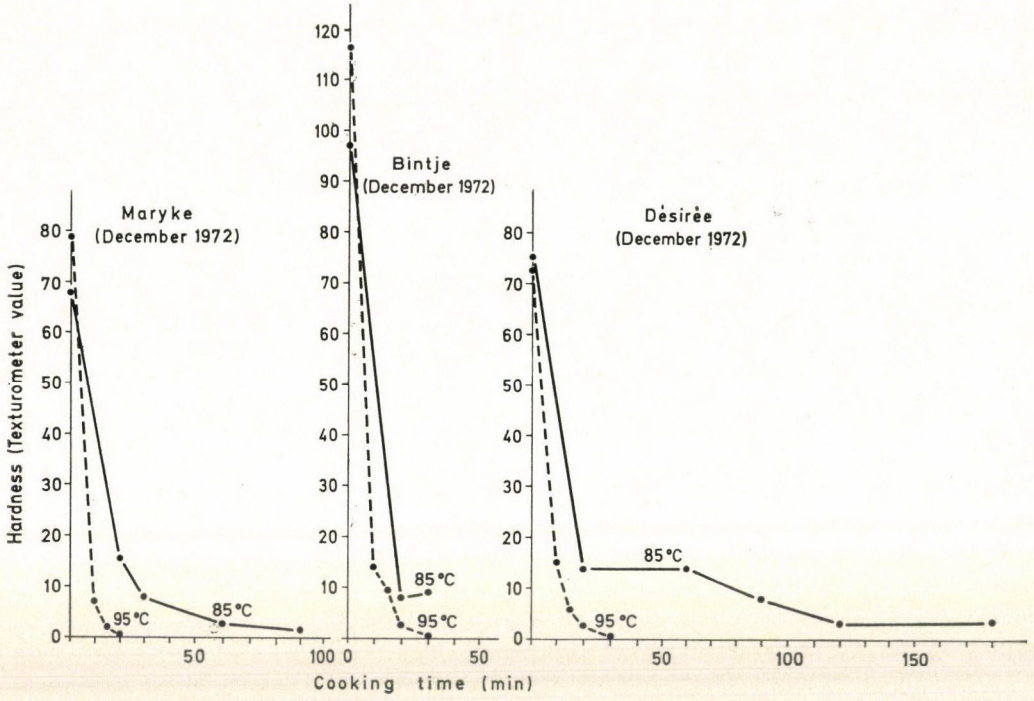

Fig. 2. Changes in the hardness of potatoes as a function of cooking time and temperature (1.5 mm thick discs). Averages of 3 parallel measurements

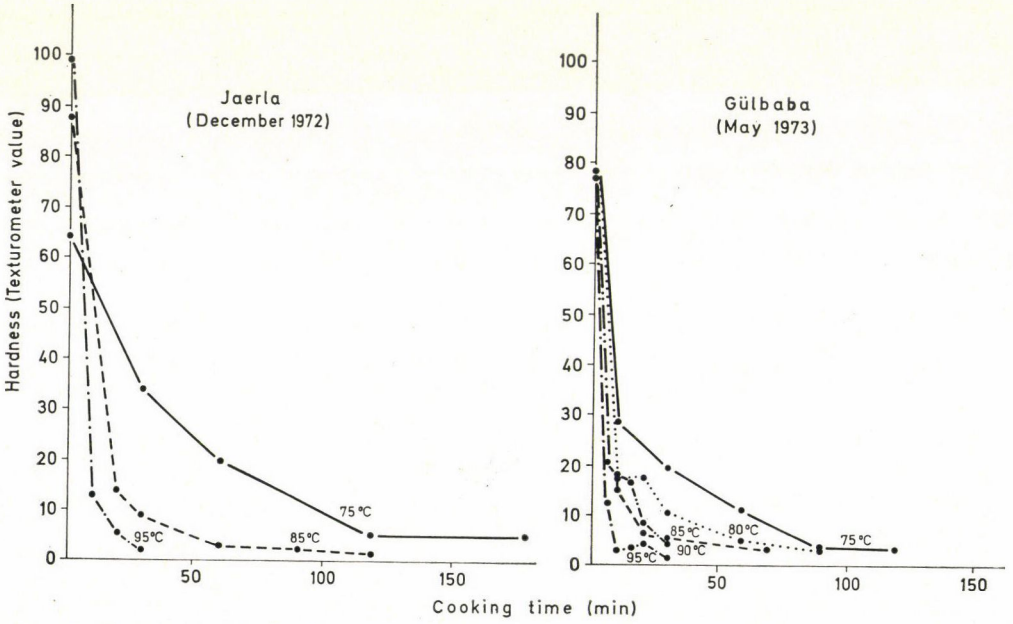

Fig. 3. Changes in the hardness of potatoes as a function of cooking time and temperature (1.5 mm thick dics). Averages of 3 parallel measurements

From these data, Q_{10} values were calculated and are shown in Table 6. Apparently, there is no distinct difference between Q_{10} values derived from cooking time requirement values pertaining to a target hardness of 50%. Also, Q_{10} does not seem to be influenced by the variety.

Plotting softening velocity against cooking temperature resulted in a straight-line relationship as illustrated in Fig. 4.

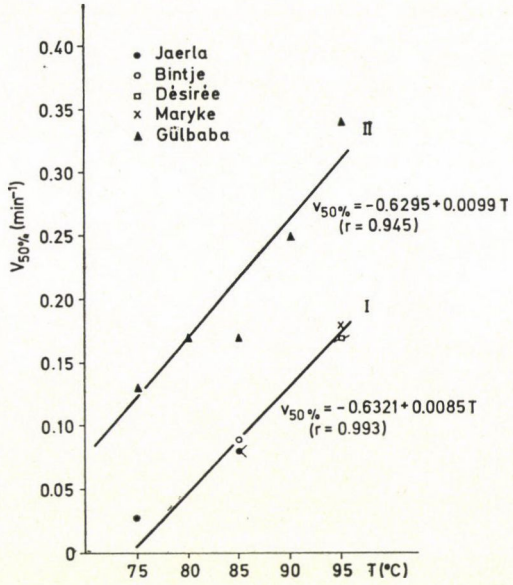

Fig. 4. Temperature (T) dependence of softening velocity ($v_{50\%}$) in potato cooking. Curve I: December 1972; Curve II: May 1973

This relationship can be used to interpolate the velocity values for any temperature between 75 and 95 °C. These, then, can be utilised to calculate the "cooking equivalent" of any given heat process with known temperature history in the range between 75 and 95 °C.

2.3. Sensory studies of the cooking process

In the course of experiments on the softening of potatoes during cooking at 95 °C, as described in para. 2.1. (15 mm thick slices), sensory tests have also been carried out to determine the cooking time necessary to reach the "cooked state" from the organoleptic point of view. The results are shown in Fig. 5.

As described in para 1.4., a 5-score system was used and score 3 was defined as the optimum texture characteristic of the properly cooked state. It can be seen that, in the November tests, 14—20 min at 95 °C were required to reach this stage, while 6 months later these values were between ca 9 and 15 min.

Table 4

Changes in consistency of potato varieties as affected by cooking temperature and time (1.5 mm thick discs, 26 mm in diametre)

Variety	Cooking time (min)	Cooking temperature (°C)									
		75		80		85		90		95	
		Texturometer hardness value									
		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Désirée	0					75.0	12.2			72.5	14.3
	6									15.1	2.1
	10									5.6	0.5
	15										
	20					13.6	3.3			2.6	0.3
	30									d	
	60										
	70										
	90					7.8	2.5				
	120					3.1	1.1				
	180					3.5	0.7				
Maryke	0					68.3	22.6			78.8	17.4
	6										
	10									7.0	2.4
	15					15.6	1.3			2.1	1.3
	20					7.8	2.3			d	
	30					2.6	0.7			d	
	60										
	70										
	90					1.5	0.4				
	120					d					
	180					d					
Jaerla	0	63.7	15.5			87.5	22.5			99.5	12.2
	6										
	10									13.0	7.6
	15										
	20					14.1	2.0			5.3	0.5
	30	34.0	7.1			8.8	2.3			2.0	0.8
	60	19.6	5.3			3.0	0.8				
	70										
	90					2.7	0.8				
	120	5.3	1.5			1.5	0				
	180	5.0	0			d					

Table 4

(continued)

Variety	Cooking time (min)	Cooking temperature (°C)									
		75		80		85		90		95	
		Texturometer hardness value									
		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Bintje	0					97.0	15.4			116.6	9.5
	6										
	10									14.3	4.5
	15									9.5	0.7
	20					8.0	3.0			2.5	0.4
	30					8.8	1.3				
	60					d					
	70										
	90					d					
	120										
	180					d					
Gülbaba	0	77.5	8.7	77.5	8.7	77.5	8.7	77.5	8.7	77.5	8.7
	6							20.1	4.9	12.0	1.7
	10	27.9	2.1	17.0	1.5	14.5	0.9	17.6	2.1	2.5	1.3
	15							16.0	1.0	3.3	2.3
	20	19.3	3.2	17.3	1.5	6.3	0.3	7.5	0.6	4.2	1.6
	30			10.1	1.9	5.3	0.6	4.3	0.6	0.8	0.3
	60	10.5	0.5	4.5	0.5			d			
	70					3.2	0.8				
	90	3.5	0.9	3.3	1.2						
	120	3.0	0.9	d							
	180	d									

d = overcooked, disintegrated
$\bar{x}$ = average
s = standard deviation
N = 3
Testing dates: Gülbaba: May 1973; other varieties: December 1972

2.4. Comparison of instrumental and sensory hardness data

Hardness, measured on the same samples with the *Texturometer* and by the sensory method, respectively, was compared in Fig. 6.

It can be seen that the correlation between *Texturometer* value and sensory score is not a very close one. It is apparent, however, that optimal sensory hardness (scores between 3.0 and 3.2) can be experienced only below a *Texturometer* hardness value of about 45. It is also evident that the instru-

Table 5

Cooking times necessary to reduce hardness to 50 and 25%, resp., of the original (raw) value in potato discs (1.5 mm thickness)

Variety	Cooking temperature (°C)	Cooking time requirement (min)		Softening velocity	
		$t_{50\ \%}$	$t_{25\ \%}$	$v_{50\ \%}$	$v_{25\ \%}$
Désirée	85	12.0	18.0	0.080	0.06
	95	6.0	9.0	0.166	0.11
Maryke	85	13.0	19.5	0.080	0.05
	95	5.5	7.5	0.182	0.13
Jaerla	75	34.0	75.0	0.022	0.013
	85	11.5	17.0	0.083	0.059
	95	6.0	8.5	0.169	0.119
Bintje	85	11.0	17.5	0.090	0.06
	95	6.0	8.0	0.166	0.125
Gülbaba	75	7.5	20.0	0.130	0.05
	80	6.0	9.5	0.170	0.11
	85	6.0	9.0	0.170	0.10
	90	4.0	7.5	0.250	0.13
	95	3.0	5.0	0.340	0.20

Testing dates: Gülbaba: May 1973; other varieties: December 1972

mental method is more sensitive than the sensory test, as the former shows differences which cannot be detected by the senses.

In Table 7 the corresponding sensory and instrumental hardness values are compiled for the varieties tested in November and May after cooking times of 0, 5, 10, 15 and 20 min at 95 °C.

The same table also contains data on the cooking time requirements pertaining to end-points *(a)* of *Texturometer* hardness values of 30 and 45, respectively, as well as *(b)* of sensory scores 3.0 and 3.2, respectively.

3. Conclusions

As is well known, cooking produces profound changes in the palatability of the potato. One of the most apparent changes occurring during heat treatment manifests itself in consistency (WOODMAN & WARREN, 1972; REICHERT & BIELIG, 1973). It is known that, at temperatures of 50—70 °C, decomposition

Table 6

Q_{10} values of potato cooking (1.5 mm thick discs)

Variety	Initial *Texturometer* value			Cooking times necessary to reach	Cooking time requirement (min)			Q_{10}	
	Cooking at				Cooking temperature (°C)				
	75	85	95						
	°C				75	85	95	85/95	75/85
Désirée	—	75.0	72.5	*Texturometer* hardness value 30	—	15.0	7.0	2.14	
				50% of original hardness	—	12.0	6.0	2.0	
Maryke	—	68.3	78.8	*Texturometer* hardness value 30	—	15.0	7.0	2.14	
				50% of original hardness	—	13.0	5.5	2.35	
Jaerla	63.7	87.5	99.5	*Texturometer* hardness value 30	38.0	15.5	8.0	1.94	2.45
				50% of original hardness	34.0	11.5	6.0	1.92	2.95
Bintje	—	97.0	116.6	*Texturometer* hardness value 30	—	15.5	8.0	1.96	
				50% of original hardness	—	11.0	6.0	1.84	
Gülbaba	77.5	77.5	77.5	*Texturometer* hardness value 30	9.5	7.25	4.0	1.98	1.30
				50% of original hardness	7.5	6.0	3.0	2.0	1.25

Testing dates: Gülbaba: May 1973; other varieties: December 1972

of the Ca and Mg complexes of pectin, the firming agent in the middle lamella of the potato tuber, occurs. Intercellular adhesion within the tissue exerts a strong effect on the decomposition of the tissue, adhesion being a function of the starch amylose, Ca, Mg and pectin contents (LINEHAN & HUGHES, 1969; PAULUS, 1971). According to WILLIAMS (1963), texture depends on the number of cells per unit surface, *i.e.*, on cell size. REEVE (1972a) showed microscopically that, on cooking, the originally angular (polyhedral) tissue cells assume a more or less "rounded-off" form and separate. Around 75 °C, gelatinization of starch sets in, this temperature value depending, *i.a.*, on the size of the starch granule (STADLER & SCHALLER, 1972). The rigidity of gelatinized starch differs from that of native starch, the latter being more resistant to pressure (REEVE, 1972b). Similar changes occur, but at a slower rate, during storage of the potato. This is clearly shown by the data presented here. After prolonged storage (November to May), substantial decrease in cooking time requirement could be observed.

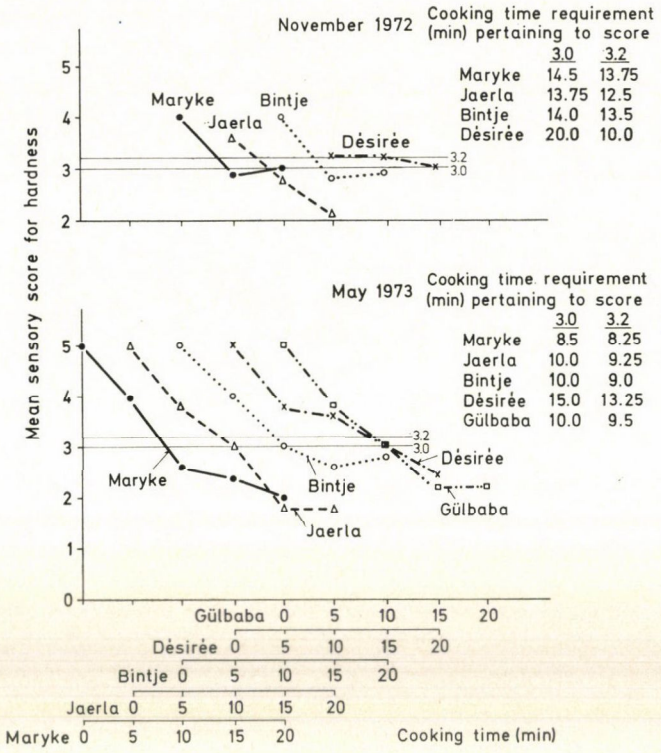

Fig. 5. Sensory evaluation of the cooked state of potatoes heat treated at 95 °C for various periods (15 mm thick slices)

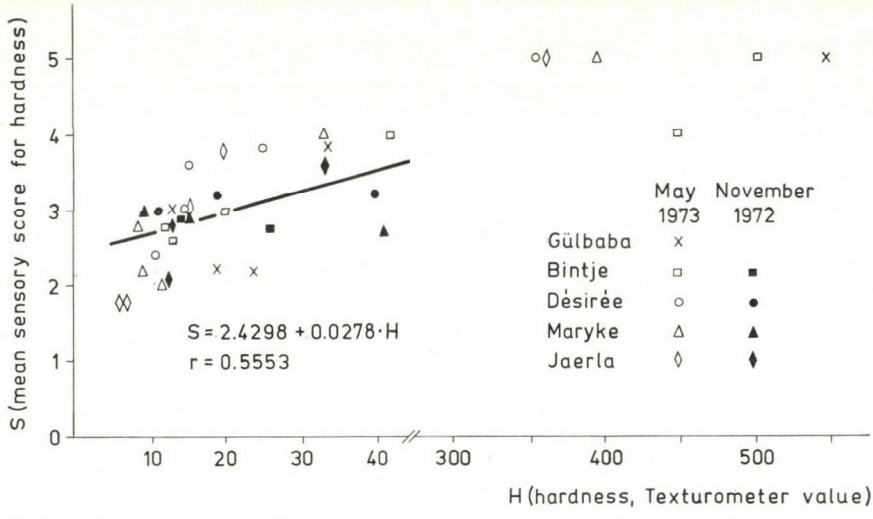

Fig. 6. Correlation of potato hardness data as measured on 15-mm slices by the *Texturometer* and by a sensory panel, respectively (May 1973)

By comparing cooking times necessary to reach a predetermined absolute hardness value (*e.g. Texturometer* value 45 or 30), certain varietal differences could be detected. The slowest cooking rate was shown by Bintje and Désirée. It is interesting to note that these varieties contain comparatively small amounts of starch and are less mealy than the other varieties tested (O.M.F.I., 1973).

In our experiments, the rate of cooking, as represented by the reciprocal of the cooking time required to reach a definite amount of change in hardness,

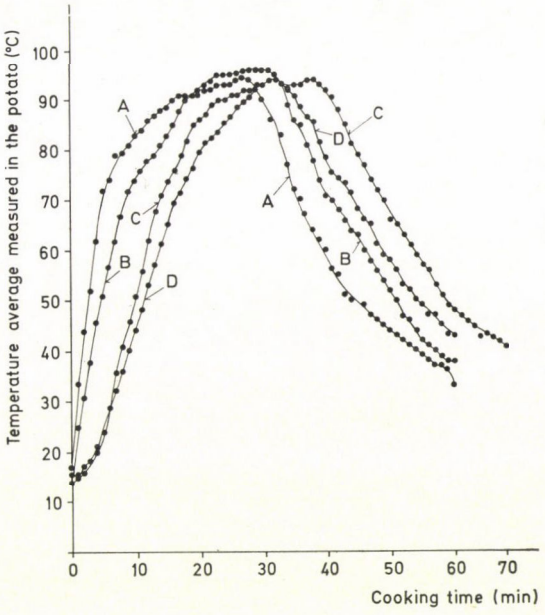

Fig. 7. Heat penetration curves measured on the surface and in the centre of "small" and "large" potato tubers of the Désirée variety. *A*: "small" tubers, surface; — *B*: "large" tubers, surface; — *C*: "small" tubers, centre; — *D*: "large" tubers, centre

was a linear function of the temperature of heat treatment. The Q_{10} values of cooking between 75 and 95 °C were found to be between 1.8 and 2.9, mostly around 2.0, corresponding to the temperature coefficient characteristic of chemical reactions in general. Neither the variety, nor the length of storage appeared to exert appreciable influence on the Q_{10} value.

It could be shown that some correlation between organoleptic and instrumental evaluation of potato hardness exists. Further, it turned out that sensory methods can only be used in a fairly narrow hardness range, the upper limit of sensory evaluation being around *Texturometer* hardness values of 50 to 60.

From the above data, the "cooking requirement" of a given heat treat-

Table 7

Comparison of instrumental and sensory evaluation of consistency changes in potato cooking (15 mm thick slices, 95 °C)

Cooking time (min)	Storage time (months)	Variety									
		Bintje		Désirée		Maryke		Jaerla		Gülbaba	
		S	H	S	H	S	H	S	H	S	H
		Consistency data									
0	0	—	415.00	—	427.10	—	358.80	—	378.90	—	—
	6	5.0	506.60	5.0	355.00	5.0	397.50	5.0	363.70	5.0	345.0
5	0	—	254.49	—	266.33	—	217.73	—	237.13	—	—
	6	4.0	42.25	3.8	25.00	4.0	33.00	3.8	19.80	3.8	33.3
10	0	4.0	81.25	3.2	39.95	2.7	41.33	3.6	32.89	—	—
	6	3.0	19.75	3.6	14.70	2.6	8.75	3.0	9.60	3.0	12.5
15	0	2.8	25.87	3.2	18.85	2.9	14.87	2.8	13.07	—	—
	6	2.6	13.00	3.0	14.60	2.4	7.80	1.8	6.00	2.2	19.1
20	0	2.9	13.88	3.0	10.75	3.0	9.31	2.1	11.97	—	—
	6	2.8	11.90	2.4	10.30	2.0	11.50	1.8	6.25	2.2	23.6
To reach		Cooking time requirement (min)									
Texturometer value 30	0		14.60		12.40		11.80		10.80		—
	6		7.75		6.75		5.50		4.25		5.50
Texturometer value 45	0		12.80		9.70		9.60		9.50		—
	6		4.75		4.50		4.00		3.25		4.25
Sensory score 3	0	14.0		20.0*		14.50		13.75		—	
	6	10.0		15.0		8.50		10.00		10.0	
Sensory score 3.2	0	13.5		10.0*		13.75		12.50		—	
	6	9.0		13.5		8.25		9.25		9.5	

S = average sensory score
H = *Texturometer* hardness value
— = not tested
* = from irregular curve

ment, applied under practical conditions of potato cooking, can be calculated as shown below.

In an experiment 3 kg potato tubers of the Désirée variety and of two size grades ["*small*": average diametres: 65 mm (long axis) and 55 mm (small axis), resp.; average weight: 87.7 g (range: 75—98 g); and "*large*": average diametres: 82 mm (long axis) and 66 mm (small axis), resp.; average weight:

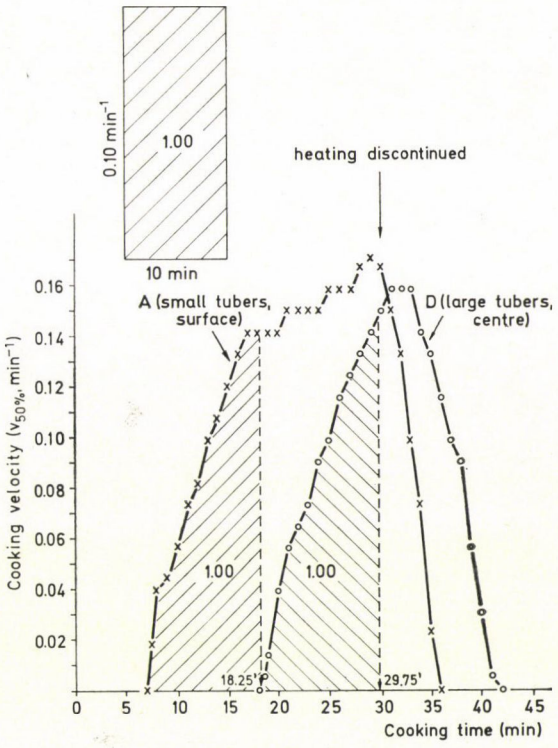

Fig. 8. Calculation of the cooking time requirement of the 5 mm deep surface portions of small tubers and the centre parts of large tubers, based on the heat penetration curves *A* and *D* in Fig. 7, and on the temperature dependence of cooking velocity (Curve I in Fig. 4). — It can be seen that a heating time of 18.25 min sufficed to soften the outside portions of small tubers to 50% of their original hardness, while it took 29.75 min to achieve the same in the centre of large potato tubers

163.0 g (range: 147—172 g)] were placed in boiling water (tuber to water ratio 1 : 2) and heated for 30 min. Then the cooking water was poured off and the temperature on the surface (5 mm deep) and in the centre (core) of the tubers was measured during both the boiling and the cooling periods for a total length of 60 min. All heat penetration measurements were performed on 5 tubers in each size group. The results are shown in Fig. 7.

From Fig. 4, velocity of softening was computed for all temperatures occurring in the tuber during heating. If, instead of the temperature data, the corresponding cooking (softening) velocities (*e.g.* v_{50} %) arep lotted against duration of heat treatment, cooking time requirement curves *A* and *D* in Fig. 8 are obtained. It can be seen that heat treatment requirements needed to reduce hardness to 50% of its original value, are met at 18.25 min of heating in the 5-mm-deep surface portion of small tubers and at 29.75 min of heating with the core portions of large tubers, since the area below the curves reaches the value of unity (1.00) at these cooking times.

It is also evident that, by applying the heat treatment described above (30 min heating + 30 min cooling), the total heat load on the tubers exceeds the actual heat treatment requirement (1.00) several times. From the total areas below the two curves it can be calculated that the surface of small tubers received a heat treatment 3.38 times larger than necessary, while the inside of large tubers was exposed to 2.24 times the heat treatment requirement defined above.

Thus, overheating could have been avoided by instantaneous forced cooling after 18.25 and 29.75 min, resp., or by so adjusting (reducing) the cooking times that the areas below the heat treatment requirement curves, covering both the heating-up and cooling-down phases, assume the value of 1.00.

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Acta Alimentaria, Vol. 4. (1), pp. 23–35 (1975)

UTILIZATION OF ALPHA-AMYLASE-SUPPLEMENTED DIET IN TURKEY FEEDING TESTS

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(Received April 5, 1973)

The applicability of an alpha-amylase enzyme preparation of microbial origin was studied in experimental turkey breeding to obtain information on the quantity of enzyme preparation which, added to the feed, would bring about a more favourable weight gain and a better utilization of the feed.

The experiments were carried out on sexed hybrid turkey males.

In the exploratory experiments the animals were given 1 000, 10 000 and 20 000 SKB units, resp., of alpha-amylase per kg of diet. With the exception of the last enzyme concentration the application of the enzyme preparation had a favourable effect on feed utilization.

In the period between the age of one and 98 days, the quantity of feed needed for a weight gain of 1 kg was reduced by enzyme addition. As compared to the value of 3.10 kg needed by the control group, enzyme supplementation at a rate of 1 000 and 10 000 SKB U kg^{-1} reduced this feed requirement by 9% (to 2.82 kg) and by 14% (to 2.67 kg), resp. (Table 3).

Hence, the experiments were continued by repeatedly administering to the animals feeds supplemented with 1 000 and 10 000 SKB units, resp., of alpha-amylase per kg of feed. Of the diets with two different enzyme concentrations, the one containing 10 000 SKB units of alpha-amylase per kg of feed was found to be the more favourable.

As compared to the control group, increase in body weight of 86-day-old animals fed enzyme supplemented diet, was 3.6% higher (3 605 g and 3 480 g, resp., significant at the probability level of 95%), while feed efficiency was improved by 4% (2.38 and 2.48 kg per kg body weight, resp., significant at the probability level of 75%; Figs. 3 and 6).

The experimental results (Table 3 and Fig. 2) indicate that it is advisable to consider the age of the animal in selecting the proper enzyme concentration. Beside feeding with the same enzyme concentration it seems necessary to investigate the effect of feeds with gradually raised enzyme activity and to determine the optimum enzyme concentration in the different periods of breeding.

Utilization of the various components of a diet in the digestive tract of the turkey involves highly complex enzymatic processes.

In the case of carbohydrates this means a degradation, as a result of which the products of hydrolysis, primarily starch derivatives, become water-soluble, so that, by being resorbed through the mucous membrane, they may reach circulation and can be utilized.

Degradation of starch in the diet is performed by

— the animal's own amylolytic enzymes;
— the enzymes in the feed;
— and, presumably, the enzyme system of the microflora introduced with the feed.

There is but little known about the amylolytic enzymes in the digestive tract of the turkey. In the saliva, amylase has been detected which displays its action in the crop (MÉSZÁROS, 1966). The enzyme activity of the crop's own secretion is a matter of contention (KEMÉNY, 1966).

The wheat, barley and oat used as feed have a beta-amylase activity and also a slight alpha-amylase activity (GREENWOOD & MILNE, 1968). The alpha-amylase activity of maize is known (KOZMINA & KRETOVICS, 1952). Although soybean meal does contain starch- liquefying enzyme (LEARMONTH & WOOD, 1960), the activity of the latter decreases depending on extraction and heat treatment. The pH of about 6.0 in the crop (SZÉP & SZEGEDI, 1963) is almost optimal for the activity of alpha-amylase of plant origin, but, due to the low enzyme concentrations, their amylolytic effect is not significant.From the crop the feed passes through the glandular stomach (pH 4.7) into the muscular stomach where the pH is 2.2 (MÉSZÁROS, 1966).

Below pH 5.0 the alpha-amylases of plant origin are rapidly and irreversibly inactivated (GREENWOOD & MILNE, 1968), so that it can be assumed that in the middle and lower intestines they do not participate in the further degradation of the feed. The feed passing from the muscular stomach into the intestinal tract is hydrolyzed there at pH 5.6 to 6.8 by the amylases of the intestinal secretion and of the pancreas.

In vitro it is possible to enhance the decomposition of starch in the diet by the addition of alpha-amylase enzyme preparations (PÁRKÁNY & HERMANN, 1969). If alpha-amylase of microbial origin is added to the diet used for breeding turkeys, enzyme concentration in the crop is being raised simultaneously. This, presumably, improves the hydrolysis of starch there. Since the optimum pH of bacterial alpha-amylase is 6.0 (MORO *et al.*, 1963; WHISTLER & PASCHALL, 1965), pH conditions in the crop are favourable for the microbial enzyme preparation, too. Apart from amylases of animal origin, the transport of microbial alpha-amylase in the intestinal system and its hydrolyzing effect might depend on its possible deactivation in the muscular stomach.

Supplementation of the diet administered to turkeys with alpha-amylase of bacterial origin has been found advantageous for animals between the age of one day and three to four weeks (MORAN & MCGINNIS, 1968), while at the age of eight to 20 weeks, the enzyme preparation had no effect (MORAN & MCGINNIS, 1966).

Taking into consideration the aforesaid, we set ourselves the target to investigate

— the possibilities of applying enzyme preparations of microbial origin in breeding turkeys;
— further, to obtain information on the enzyme concentration to be added to diets.

1. Materials and methods

1.1. Materials

1.1.1. Animals. The experiments were carried out with sexed hybrid turkey males bred by the *Poultry Research Institute* at Gödöllő (Hungary). The animals were marked on their wings.

1.1.2. Diets. In the course of the experiments two types of diet of different composition were given to the animals. From the age of one day to that of 28 days they were administered so-called initiating turkey diet, from the age of 29 to 98 or 84 days, *i.e.*, up to the end of the experiment, they were fed breeding turkey diet. In all series of experiments the diet was prepared from the same base material. Grained products were comminuted with the hammer mill type D-24 (rpm 2 960). The components and the enzyme preparation were mixed on the homogenizer type *Agrostroj Galanta*, Czechoslovakia. Homogeneous distribution of the enzyme preparation was checked by determination of the enzyme concentration of samples of the ready diets. The components of the diets are shown in Table 1.

Table 1

Composition of initiating and breeding turkey diets

Components of diet	Initiating diet, %	Breeding diet, %
Maize	41.00	30.00
Barley	4.00	—
Oat	3.00	—
Wheat	—	28.50
Wheat bran	3.50	—
Extracted soybean meal	22.00	25.00
Alfalfa meal	5.00	—
Milk powder	2.00	—
Fish meal (70% protein)	12.00	10.00
Animal feeding yeast	3.00	1.00
Dicalcium phosphate	0.50	1.75
Feeding limestone	2.00	1.50
Feeding salt	0.40	0.25
Vitamin premix	1.00	1.00
Mineral premix	0.60	1.00
Total, %	100.00	100.00

1.1.3. The enzyme preparation. A powdered preparation of *Bacillus subtilis* origin, *Bakterie Amylase Novo 264*, manufactured in Denmark, was used in the experiments. The optimum pH of the enzyme preparation was between 5.7 and 7.0, its optimum temperature between 70 and 85 °C. The preparation kept its activity in the pH range between 3.0 and 10.0 (as quoted from the leaflet of the manufacturing firm).

1.2. Methods

1.2.1. Breeding conditions. After sexing, the 1-day-old turkeys were transferred to the brooding house. Breeding conditions were the same in all three experiments, but the tests were carried out in different brooding houses and at different times. In the first series four compartments of a large multi-compartment brooding house were included in the test, while for the second and third experiment 16 compartments were made use of. The density of the stock was five animals per square meter.

A brooder stove with parabolic mirror was placed into each compartment. In the first 14 days the brooder stove was fenced off to ensure that the young animals stay only in the heat-irradiated area. Programming of temperature and of illumination in the period of growing are given in Table 2.

Table 2

Programming of temperature and of illumination in the various periods of breeding

Time (days)	Temperature under the brooder stove (°C)	Room temperature (°C)	Illumination	
			hours	light intensity (Wm^{-2})
0— 7	35—37	26	24	3
8—14	33	24	22	2
15—21	30	22	17	2
22—28	27	22	17	2
29—35	—	22	17	2
36—42	—	20	17	2

From the sixth week up to the end of the experiment the temperature and illumination of the brooding house was the same.

Ventilation was provided for the age group of 1 to 56 days by 5.5 m^3, for the age group of 56 to 84 days by 7.5 m^3 air per hour per 1 kg of body weight.

In the first experiment the breeding period lasted from the age of one

day to that of 98 days, in the next two experiments from the age of one day to that of 84 days.

During the experiment the turkeys consumed diet *ad libitum*.

1.2.2. Data processing. Weight gain and feed consumption were checked, the first by weighing each group every fortnight and each individual at the end of the experiment, the second was determined every fortnight for each group.

From the experimental data the

— average body weight

— and specific feed utilization of the animals were obtained by calculation.

Specific feed utilization is defined as the quantity of diet used up for the production of 1 kg of body weight and was always calculated from the age of one day up to the end of the experiment.

1.2.3. Determination of alpha-amylase concentration. The concentration of bacterial alpha-amylase in the enzyme preparation, used throughout the experiments and in the diet administered to the animals, was determined by *Wohlgemuth*'s method as modified by SANDSTEDT and his co-workers (SANDSTEDT *et al.*, 1939) and expressed in SKB units.

1.2.4. Mathematical-statistical processing of experimental data. Body weight and feed consumption of animals bred on feeds of various enzyme concentrations were compared by means of *Student*'s t test to the corresponding data of stock kept on enzyme-free diet.

Because of the fairly great scattering of experimental data, differences between the arithmetical average of the control group and of the group fed on enzyme-containing feed, were evaluated at the 75% probability level, too.

In addition, when evaluating individual body weight data at the end of the experiment, the empirical and theoretical frequency distributions in the respective groups kept on enzyme-free diet and on diet containing 10 000 SKB units per kg were compared by graphical fitting.

2. Results

2.1. Preliminary experiment

In the experiment designed to furnish preliminary information, 200 one-day-old turkeys of 55 g starting weight were divided into four groups. One group was given enzyme-free feed, while to the feed of the other three groups 1 000, 10 000 and 20 000 SKB units, resp., of microbial alpha-amylase per kg of feed were mixed.

Average body weight of the turkeys and amounts of diets needed for a 1-kg gain during the period of breeding are shown in Table 3.

Table 3

Average body weight and specific feed utilization of turkeys kept on feeds of enzyme-free diet and of different enzyme concentrations

Time of weighing, days	Bacterium alpha-amylase concentration in the feed (in SKB units per kg)							
	0		1 000		10 000		20 000	
	A	B	A	B	A	B	A	B
1st	55	—	55	—	55	—	55	—
14th	239	1.43	234	1.27	239	1.31	239	1.28
28th	541	1.96	539	1.89	549	1.66	532	1.92
42nd	1 178	1.97	1 124	1.99	1 093	1.88	1 177	1.98
56th	1 764	2.42	1 765	2.00	1 800	1.97	1 839	2.21
70th	2 823	2.48	2 825	2.18	2 893	2.13	2 929	2.32
84th	3 882	2.71	3 919	2.47	3 872	2.50	3 832	2.79
98th	4 830	3.10	4 800	2.82	4 739	2.67	4 800	3.26

A = average body weight in g
B = feed consumption per 1 kg body weight in kg kg^{-1}
Batch number of the enzyme preparation: 1067
Each group contained 50 sexed turkey males

According to the data of Table 3, at the end of the breeding period the average body weight of all three enzyme-fed groups was 30 to 90 g less than that of the control group. The difference between the average body weight of the control group and of the enzyme-fed groups was not significant.

Comparison of the respective effects of enzyme-free and enzyme-supplemented diet showed no improvement of growth by alpha-amylase, although feed efficiency was increased.

Turkeys bred on diet containing 1 000 and 10 000 units, resp., of bacterial alpha-amylase needed less feed for the production of 1 kg of body weight during the entire period of breeding from the age of one day to the time of each weighing than the control animals bred on enzyme-free feed.

At the end of the breeding period, *i.e.*, at the age of 98 days, the experimental animals have consumed 280 g (or 9%) and 430 g (or 14%) less feed for the production of one kilogram of body weight than the control group.

The group given 20 000 units of enzyme per kg of feed, on the other hand, needed 160 g (or 5%) more feed than the control group for the production of 1 kg of body weight. Since the data were obtained for groups of animals, it was not possible to evaluate significance of differences in feed utilization mathematically.

2.2. Experiments with diet containing 1 000 SKB units of alpha-amylase per kg

Starting from the results of the preliminary experiments, the next tests were carried out by giving 8 groups of turkeys diet without enzyme and 8 groups diet containing 1 000 SKB units per kg. There were 65 animals in

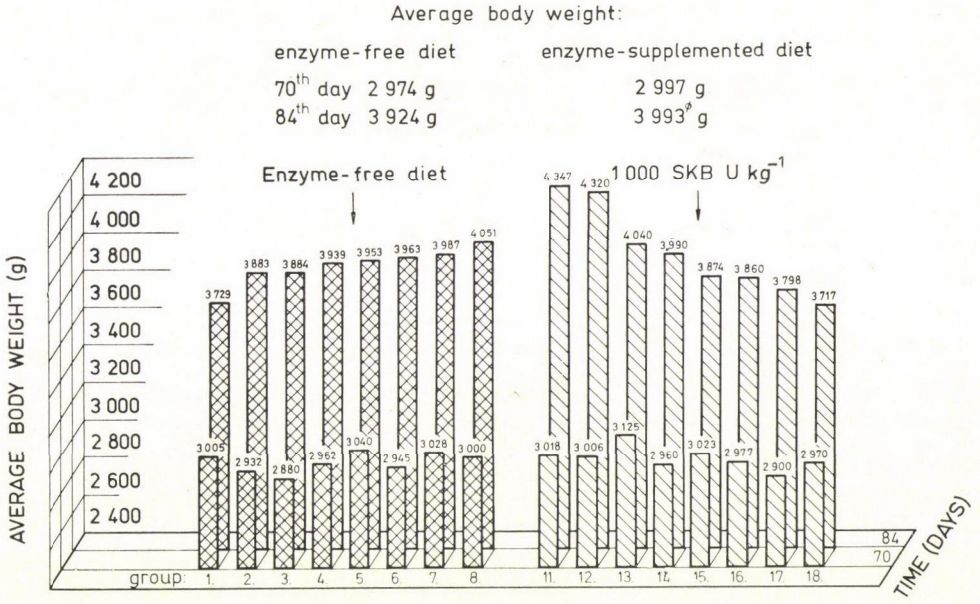

Fig. 1. Weight gain in the various groups of turkeys, kept on enzyme-free diet and on feed containing bacterial alpha-amylase, resp. Batch number of the enzyme preparation: 1 067. Each group consisted of 65 sexed, 1-day-old turkey males. ø: $P < 75\%$

each group, thus a total of 1 040 one-day-old birds of 55 g average body weight were included in the experiment.

The breeding parameters of the stock kept for 84 days were also checked every fortnight.

Up to the age of 42 to 56 days, the average body weights in the groups kept on two different diets were almost the same, hence in Fig. 1 only the results of the body weight determinations of the 70- and 84-day-old animals are shown, resp.

The average body weights of the control groups were lower both at the age of 70 and of 84 days than the body weights of animals bred on enzyme-containing diet. At the age of 70 days the average body weight of the stock kept on alpha-amylase-containing diet was 23 g higher (2 997 g instead of

2 974 g), at the age of 84 days 69 g higher (3 993 g instead of 3 924 g) than the average body weight of animals fed with enzyme-free feed.

The average specific feed utilization of the groups kept on different diets at the various times of weighing are summarized in Fig. 2. It appears from the histograms that, with the exception of 56- and 70-day-old animals, those kept on enzyme-containing diet utilize their feed more efficiently than the control group. By the end of the experiment 100 g less of the enzyme-containing feed was required for the production of 1 kg of body weight than of the control feed (2.46 kg instead of 2.56 kg).

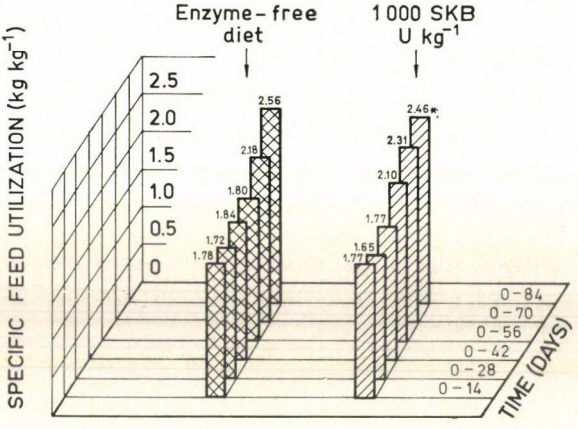

Fig. 2. Specific feed utilization in the various groups of turkeys, kept on enzyme-free and on bacterial alpha-amylase-containing diet, resp. Batch number of the enzyme preparation: 1 067. Each group consisted of 65 sexed, 1-day-old turkey males. Total number of animals at the end of the experiment: 453 and 501, resp. *: $P \leq 75\%$

Though the favourable effect of bacterial alpha-amylase is also manifest in the breeding parameters (average body weight, feed utilization) of the 84-day-old turkeys kept on feed containing 1000 SKB units per kg, this cannot be confirmed by mathematical-statistical calculations.

2.3. Experiments with diet containing 10 000 SKB units of alpha-amylase per kg

In the third experiment enzyme concentration was raised to 10 000 SKB units per kg. The test involved again 8 groups of 45 turkeys each for the enzyme-free diet and similar eight groups for feeding a diet containing 10 000 SKB units of enzyme per kg.

Similarly to the preceding experiment, the average body weights determined on the 70- and 84-day-old stock are presented graphically (Fig. 3).

The data of group tests demonstrate the higher weight gain in the enzyme-fed group as compared to the control group, both at the age of 70 and of 84 days.

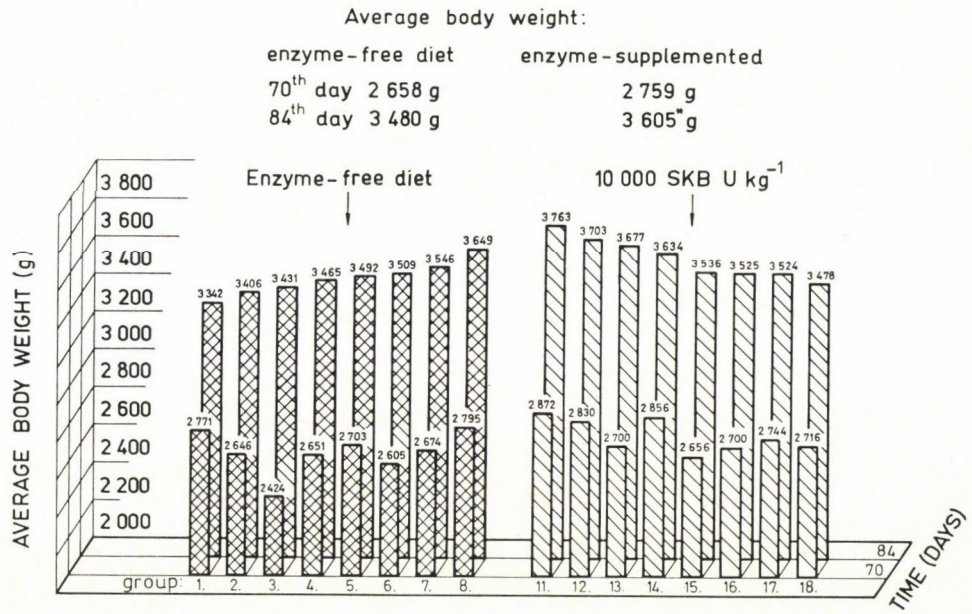

Fig. 3. Weight gain in the various groups of turkeys, kept on enzyme-free diet and on bacterial alpha-amylase-containing feed, resp. Batch number of enzyme preparation: 1 212. Each group consisted of 45 sexed, 1-day-old turkey males. *: $P \geq 95\%$

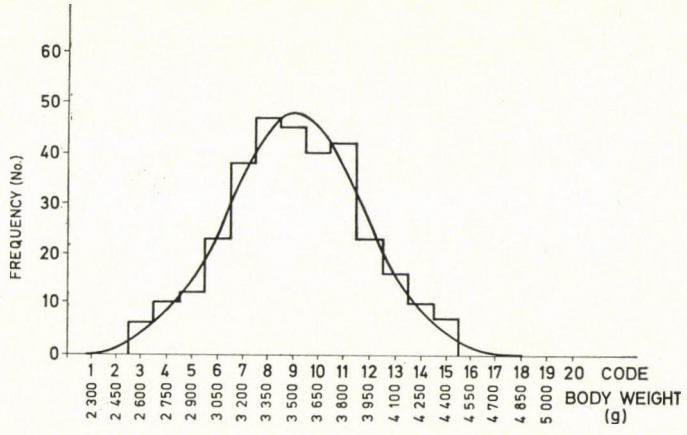

Fig. 4. Fitting of empirical frequency distribution of weights of turkeys bred on enzyme-free feed, to the normal distribution. Maximum of the normal distribution: 48.0 animals. Average body weight calculated from the normal distribution: 3 511.0 g. Mean deviation calculated from the normal distribution: 398.0 g. Number of individuals: 319

Compared to the animals kept on the control diet, at the age of 70 days the average body weights in the 8 enzyme-fed groups were 100 g higher (2 758 instead of 2 658 g), at the age of 84 days 125 g higher (3 605 instead of 3 480 g) than the average body weights of the 8 control groups. The 3.6 % excess weight of the 8 enzyme-fed groups over the average weights of the 8 groups kept on enzyme-free diet is significant at the 95 % level of probability.

Classifying the individual body weight data of the stocks kept on two types of diets at the age of 84 days into groups, their respective empirical frequency distributions did not show significant deviations from the normal distribution.

Figs. 4 and 5 illustrate graphically the fitting to the normal distribution of the empirical frequency distribution of body weights of the stock kept on enzyme-free feed and on feed containing 10 000 SKB units of enzyme per kg, resp.

Average specific feed utilization in the groups kept on different diets is shown in Fig. 6.

The favourable effect of the application of alpha-amylase on specific feed utilization is apparent at the age of 70 and 84 days, resp.

Compared to the turkeys bred on enzyme-free feed, the animals given enzyme-containing feed up to 84 days needed 100 g less feed for the production of 1 kg of body weight (2.38 kg instead of 2.48 kg) and 70 g less feed from the age of one day to the age of 70 days. A 4 % saving in feed is ensured at the 75 % probability level.

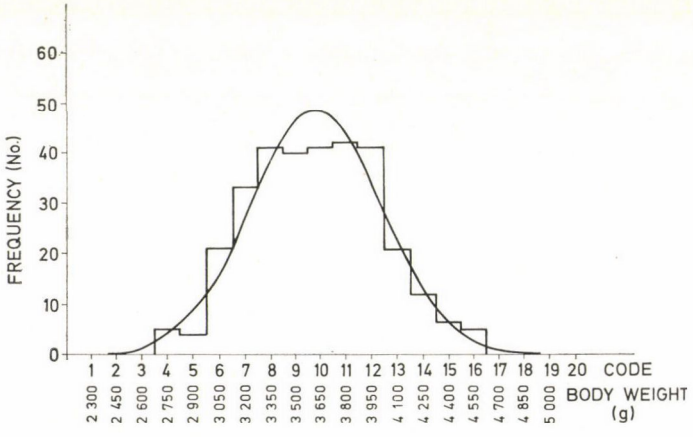

Fig. 5. Fitting of empirical frequency distribution of weights of turkeys, bred on bacterial alpha-amylase-containing feed, to the normal distribution. Enzyme concentration 10 000 SKB units per kg of feed. Maximum of the normal distribution: 48.7 animals. Average body weight calculated from normal distribution: 3 623.0 g. Mean deviation calculated from normal distribution: 384.0 g. Number of individuals: 313

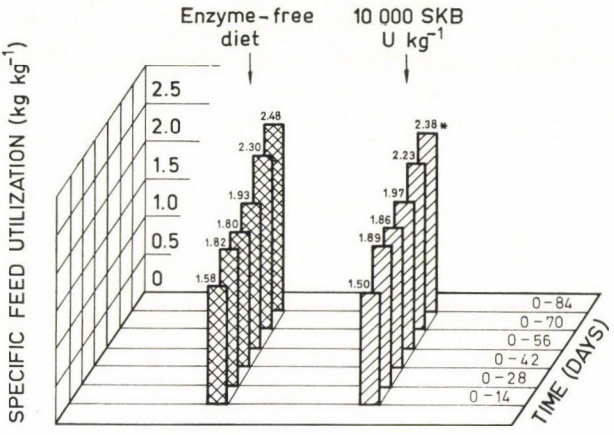

Fig. 6. Specific feed utilization of turkeys, bred on enzyme-free diet and on bacterial alpha-amylase-containing feed, resp. Batch number of enzyme preparation: 1 212. Total number of 84-day-old animals: 319 and 313, resp. *: $P \geq 75\%$

3. Conclusions

The experiments described were aimed at promoting the degradation of starch in the diet by the application of alpha-amylase of bacterial origin.

An exploratory experiment indicated a favourable effect of feeds containing 1 000 and 10 000 SKB units of enzyme per kg on the utilization of the feed. From the age of one day up to the age of 98 days 9 % and 14 %, resp. less alpha-amylase-supplemented feed was needed for the production of 1 kg of body weight than of the feed containing no enzyme. Breeding parameters were less favourable when the feed contained 20 000 SKB units of enzyme per kg (Table 3).

Consequently, in the next experiments diets were repeatedly used which contained 1 000 and 10 000 SKB units per kg, resp., from the age of one day up to the age of 84 days.

The weight surplus of turkeys bred on feed containing per kg 1 000 SKB. units of alpha-amylase was not more than 2 %. When using feed with an enzyme concentration of 10 000 SKB units per kg, the weight surplus compared to the control group was 3.6 %, confirmed on the 95 % probability level (Figs. 1 and 3).

From the age of one day up to the age of 84 days the specific feed utilization was by 4 % more favourable both for feeds containing 1000 and 10 000 SKB.

Because of the excessive scattering of the results, this 4 % saving in feed per kg of body weight for the groups kept on feed supplemented with 1 000 SKB units of alpha-amylase per kg could not be confirmed by mathematical-

statistical methods. The better feed utilization obtained by using 10 000 SKB units of alpha-amylase per kg proved significant at the 75% probability level as compared to the control group.

In the experiments carried out so far, a more favourable change in breeding parameters was found when the diet contained alpha-amylase in the concentration of 10 000 SKB units per kg. It must, however, be added that the feed contained the same amount of enzyme during the entire period of each experiment. Part of the results seems to indicate that it might be possible to reduce the quantity of the enzyme preparation in the first half of the breeding period. Apparently, for the young growing birds it might be superfluous to add larger quantities of alpha-amylase to the feed.

In the first half of the breeding period the turkeys were able to make good use of the feed with lower enzyme concentration (1 000 SKB units per kg) for body weight building.

According to the data of the first experiment, from the age of one day up to the age of 56 days, the administration of the enzyme preparation helped to reduce the feed needed for the production of 1 kg of body weight from 2.42 kg to 2.00 kg (Table 3). Even after the age of 56 days the utilization of feed containing alpha-amylase was more favourable than that of the control feed. At the age of 98 days 9% less was needed from the feed containing the enzyme preparation than from that without enzyme for the production of 1 kg of body weight.

In the following experiment, when the feed contained again 1 000 SKB units per kg, with the exception of the weights on the 56th and 70th day, a better utilization of the feed was observed from the 28th day on wards (Fig .2). From the age of one day up to the age of 42 days 3.8% less alpha-amylase-containing feed than enzyme-free feed was needed for the production of 1 kg of body weight (1.77 kg instead of 1.84 kg). At the end of the experiment the use of the enzyme preparation reduced specific feed utilization by 4%.

The average body weights of turkeys kept on the two different diets (Figs. 1 and 3) show that even when more alpha-amylase (10 000 SKB units per kg) is added to the feed, in the second half of the breeding period, from the age of 70 days, the body weight of the stock was 100 g and later even 125 g higher than that of the control stock.

In the first half of the breeding period the addition of the enzyme preparation to the feed had a favourable effect not so much on body weight as on specific feed utilization.

It seems advisable in forthcoming experiments to consider the age of the stock and to increase accordingly and gradually the enzyme content of the feed during the breeding period.

Further experiments might contribute to the elaboration of a technology whereby enzyme preparations of microbial origin are added to the feed of

turkeys as well as to the determination of the optimum enzyme concentration at the beginning and during breeding.

*

The author wishes to express her thanks to Dr. M. TÓTH, Deputy Director of the *Poultry Research Institute* (Gödöllő) and to Dr. M. PERÉNYI, Senior Scientist of the same institute for their valuable help in the performance of the experiments. Thanks are also due to Dr. K. MIHÁLYI, Miss A. HERMANN and Miss P. CSEPREGI of the *Central Food Research Institute* for their contribution to the experimental work.

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Acta Alimentaria, Vol. 4. (1), pp. 37—61 (1975)

FACTORS AFFECTING MEASUREMENT OF GLUCOSE OXIDASE ACTIVITY OF COMMERCIAL ENZYME PREPARATIONS

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(Received May 9, 1973)

Examination of the enzyme-catalyzed reaction leading from glucose to gluconic acid, showed that, with a glucose oxidase (GO; E.C. 1.1.3.4.) preparation (KÉKI No. 1), increasing the substrate concentration up to 0.1 *M*, increases the reaction rate considerably. Between glucose concentrations of 0.1 and 1.0 *M*, however, this response is rather sluggish. The value of the *Michaelis* constant was measured with three Hungarian preparations and found to be 0.0186, 0.0099 and 0.0122 *M*, resp. For practical activity measurement, glucose concentrations between 0.15—0.20 *M* (3—4% glucose monohydrate) appeared to be suitable.

It was observed that, with different commercial GO preparations, the correlation between activity values as expressed in *Sarrette* units (SU), and the kinetic units recommended by the IUB as expressed in μmole min^{-1}, is very poor.

In order to find the explanation for this inconsistency, the role of the usual catalase "contamination" of commercial GO preparations was first studied. With exceptions, preparations of higher catalase: GO ratio appeared to be better catalysts of glucose conversion at the same SU level. Addition of various (liver, bacterial, fungal) catalase preparations to the reaction mixture at several concentration levels showed that the rate of gluconic acid formation is strongly enhanced by catalase preparations of fungal origin, while liver or bacterial catalases were less effective.

Further, it was found that the rate of the above reaction could be decreased by the addition of the products of the reaction, *i.e.*, H_2O_2 or δ-gluconolactone. The inhibitory effect of δ-gluconolactone is less than that found in the case of H_2O_2.

Glucose oxidase is an enzyme often used in the food industry as an additive to food products and beverages. (BARTON *et al.*, 1957; OHLMEYER, 1957). The enzyme is a specific reagent for detecting glucose. It is widely used in medical laboratories for the examination of the glucose content of blood and urine (FROESCH & RENOLD, 1956) and also in various laboratories to determine the glucose concentration of sugar syrups containing several kinds of sugar (WHISTLER *et al.*, 1953).

The literature contains many data on the properties, mode of action (COULTHARD *et al.*, 1945; KEILIN & HARTREE, 1948, 1952; KUSAI *et al.*, 1960; HLAING *et al.*, 1961; PAZUR & KLEPPE, 1964) and the structure of this enzyme (KUSAI *et al.*, 1960; BODMANN & WALTER, 1965; SWOBODA & MASSEY, 1965; NAKAMURA & FUJIKI, 1968; YOSHIMURA & ISEMURA, 1971).

In the present paper the glucose oxidation rates of three glucose oxidase preparations, products of different firms, were compared with the enzyme

preparations produced in our Institute. The effects of substrate concentration and of enzyme concentration, on the reaction rate of glucose oxidation have been examined. Product inhibition was also studied.

The effect of catalase preparations on the rate of the reaction catalysed by glucose oxidase has similarly been tested.

It was finally attempted to clarify the differences between results of activity measurements based on the traditional concepts and on the kinetic method advocated by the I.U.B., respectively.

1. Materials and methods

1.1. Assay of glucose oxidase activity

The activity of glucose oxidase has been measured by titrimetric determination of gluconic acid formed from glucose by the enzyme (UNDERKOFLER, 1958; PROSZT, 1963).

For the examinations 3% glucose dissolved in acetate buffer, pH 5.1, was used as a substrate. The determinations were carried out in 500-ml Erlenmeyer flasks. The volume of the reaction mixture was 29 ml (substrate: 25 ml, plus enzyme solution, adjusted to 29 ml with distilled water). The reaction mixtures were aerated on a rotary shaker (rpm: 330; stroke: 20 mm; O_2 supply: 19 mmole l^{-1} h^{-1} determined by the method of COOPER and his co-workers (1944); temperature: 28°C) for 2.5 hours. The action of the enzyme was followed by titrimetric determination of gluconic acid in three parallels.

Reaction mixtures containing heat-inactivated enzyme were used as the blanks. At the end of the reaction period the reaction was stopped by the addition of measured amounts of 0.1 *N* NaOH. The excess alkali was titrated with 0.05 *N* HCl. From the quantity of hydrochloric acid consumed, the amount of gluconic acid was calculated and the results were given in micromoles.

The enzyme activity stated in the traditional *Sarrette* units was determined according to the following formula:

$$[\text{blank (0.05 } N \text{ HCl ml)} - \text{sample (0.05 } N \text{ HCl)}] \cdot 1.5.$$

1.5 is a factor, with the use of which the results obtained by the titrimetric method can be converted into *Sarrette* units as determined manometrically.

Sarrette unit (SU): this is the amount of enzyme that can cause an O_2 uptake of 10 mm^3 min^{-1}, in a *Warburg* manometer at a temperature of 30°C.

For the kinetic examinations gluconic acid content of the reaction mixtures was determined by the titrimetric method at 30-minute intervals for 4 hours.

The enzyme preparations used for the kinetic examinations were compared on the basis of their reaction rate (μmole gluconic acid per minute) calculated from the linear part of the reaction curve.

1.2. Assay of catalase activity

The activity of catalase was determined by the method of *Baker* (in: SCOTT & HAMMER, 1960). The unit (BU) of enzyme activity is the amount of enzyme which decomposes 264 mg of H_2O_2 under specific circumstances.

The ratio of catalase to glucose oxidase activity was expressed by the R value, the ratio of *Baker* units and *Sarrette* units (R = BU/SU).

1.3. Glucose oxidase preparations used

(a) SIGMA (II) preparation (14 700 SU g^{-1}; 2 440 BU g^{-1});
(b) MERCK (10 000) preparation (15 096 SU g^{-1}; 4 280 BU g^{-1});
(c) NBC (1 500) preparation (1 500 SU g^{-1}; 120 BU g^{-1});
(d) Preparations of the Enzyme Pilot Plant of the *Central Food Research Institute* (KÉKI) Budapest (ZETELAKI, 1966, 1969; MORVAINÉ & VÁMOSNÉ, 1969).

The enzymes were produced in submerged culture by an *Aspergillus niger* strain in 100-liter stainless steel fermentors (agitation: 300 rpm; aeration: one $l \cdot l^{-1}$ min^{-1}; oxygen dissolution rate: 85 mmole O_2 l^{-1} h^{-1}; media, cultivation conditions and preparation of the enzymes are given in previous papers (ZETELAKI & VAS, 1968; ZETELAKI, 1969). After extraction of the enzyme from the culture filtrate, the crude enzyme preparations were precipitated with isopropyl alcohol. Their designation and characteristics are enumerated below:

KÉKI No. 1	5000 SU g^{-1};	500 BU g^{-1}
KÉKI No. 3	7190 SU g^{-1};	900 BU g^{-1}
KÉKI No. 5	1300 SU g^{-1};	160 BU g^{-1}
KÉKI No. 8	13000 SU g^{-1};	300 BU g^{-1}
KÉKI GT. (purified)	37 200 SU g^{-1};	0 BU g^{-1}
KÉKI No. 72/9	6 400 SU g^{-1};	1 400 BU g^{-1}
KÉKI No. 72/10	5 800 SU g^{-1};	1 400 BU g^{-1}

1.4. Catalase preparations used

Liver catalase (SIGMA C-10)	12 000 BU g^{-1}
Bacterial catalase (MERCK)	8 000 BU g^{-1}
Penicillium catalase [Ukr.N.I.I.S.L.P. (Ukrainian Scientific Research Institute of the Distilling and Liqueur Industry) Kiev]	10 000 BUg^{-1}
Aspergillus catalase [U.V.U.P.P. (Central Food Research Institute, Prague)]	200 BU g^{-1}

2. Results

2.1. The effect of concentration of substrate on the reaction rate

In the course of our work the effect of substrate concentration (0.025, 0.05, 0.1, 0.2 and 1.0 *M*) on the glucose oxidation rate of the glucose oxidase preparation KÉKI No. 1 (specific activities: 5 000 SU g^{-1} and 500 BU g^{-1}; R = 0.100) was investigated. The concentration of the enzyme preparation in the reaction mixtures was 0.038 mg ml^{-1}, while activities of the reaction mixtures were 0.190 SU ml^{-1} and 0.019 BU ml^{-1}, resp. The reaction was followed for 240 min. The glucose conversion of the above preparation at various substrate concentrations was plotted against the reaction time in Fig. 1. The reaction rates for different substrate concentrations were calculated by regression analysis from the linear part of the curves. The reciprocals of the reaction rates were plotted as functions of the reciprocal of substrate concentration in Fig. 2.

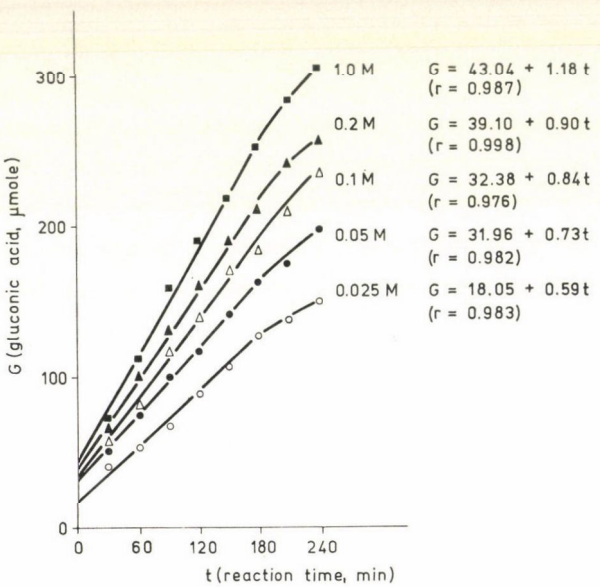

Fig. 1. Effect of substrate concentration on the rate of oxidation of glucose by a glucose oxidase preparation, KÉKI No. 1. (Experimental points represent the mathematical means of three parallels. Coefficients of variation were between 4.0 and 6.5 per cent in the case of the parallels of all five curves. Equations were calculated by regression analysis from the linear part of the curves.)

As can be seen in Fig. 1 the increase in substrate concentration resulted in an increase of the reaction rate, but substrate concentrations higher than 0.2 *M* had diminishing influence on reaction rate.

The values of the *Michaelis* constant (K_m) and the V_{max}, calculated by regression analysis from the above reaction rates of glucose oxidase preparation KÉKI No. 1, proved to be 0.0186 *M* and 1.123, resp. (Fig. 2).

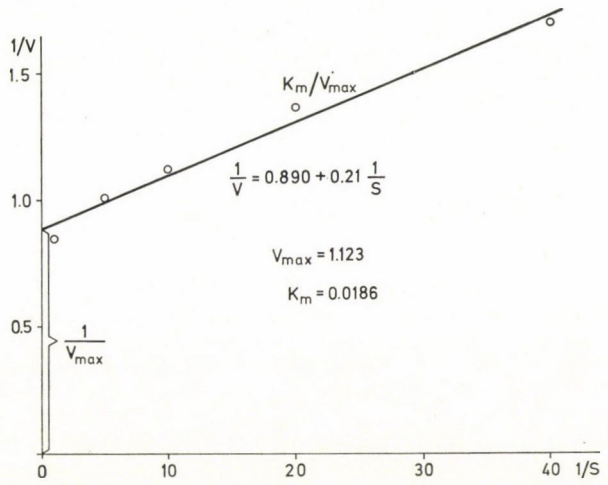

Fig. 2. Plot of 1/V [V = gluconic acid formed (μmole min^{-1})] against 1/S [S = glucose (mole)]. Glucose oxidase KÉKI No. 1. Enzyme activities of the reaction mixtures: 0.190 SU ml^{-1} and 0.019 BU ml^{-1}. The ratio of catalase to glucose oxidase: R = 0.100

2.2. The effect of enzyme concentration on the rate of glucose oxidation

In the course of the kinetic analysis glucose oxidase preparations were examined in three different concentrations. Glucose oxidase and catalase activities of the preparations, the ratio of their catalase–glucose oxidase activity (R), as well as the enzyme concentrations applied are summarized in Tables 1 and 2.

The amounts of gluconic acid formed by the different enzyme preparations were plotted against reaction time (Figs. 3 and 4) in cases of all three adjusted activity levels (I, II and III) of the reaction mixtures (Tables 1 and 2).

As can be seen in the figures, the reaction rate is not linear throughout the whole reaction period observed. The linear phases of the curves shorten with the increase in glucose oxidase activities of the reaction mixtures.

In Fig. 5 the reaction rates of the above preparations are plotted as a function of the glucose oxidase activity (in SU) of the reaction mixtures. The reaction rates are summarized in Tables 1 and 2.

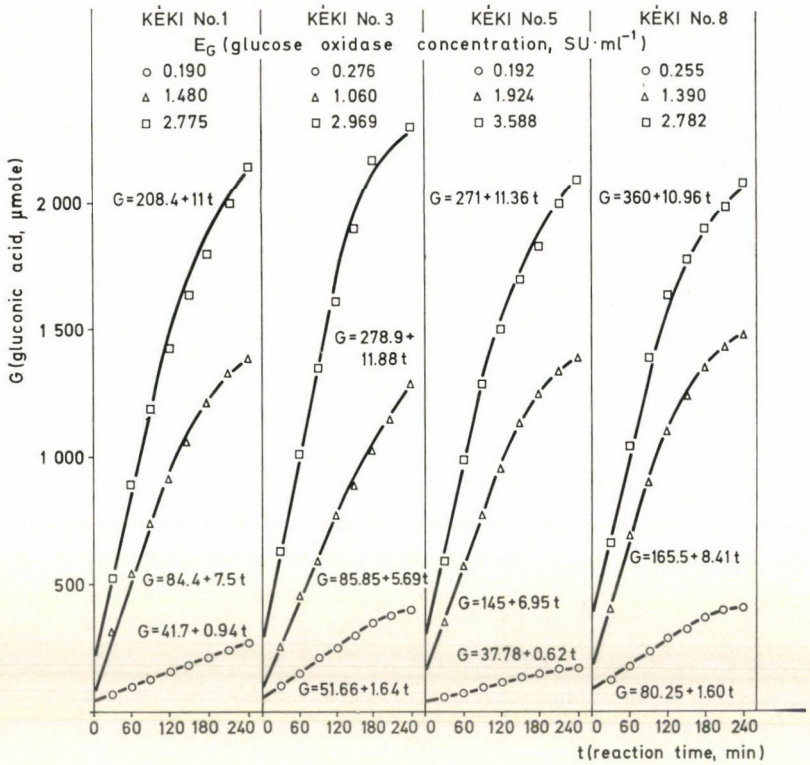

Fig. 3. Effect of enzyme concentration (E_G) on rate of glucose oxidation by some glucose oxidase preparations (KÉKI) with different R values. (The activities of the enzymes and the reaction mixtures are given in Table 1. Each experimental point represents the mathematical mean of three parallels. Equations were calculated by regression analysis from the linear part of the curves.)

Table

The effect of glucose oxidase concentration on reaction rate. The ratio of catalase to glucose rates of their

Activities of the preparations tested: KÉKI No. **1** 5000 SU g^{-1} and 500 BU g^{-1}; KÉKI No. **8** 13000 SU g^{-1} and 300 BU g^{-1}. Reaction rate was calculated from the linear part and

Enzyme preparations		Concentrations of weighed-in enzyme preparations as well as of catalase in the reaction					
		I			II		
KÉKI No.	ratio of catalase to glucose oxidase (R)	mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})	mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})
1	0.100	0.039	0.190	0.019	0.296	1.480	0.148
3	0.125	0.038	0.276	0.035	0.148	1.060	0.133
5	0.123	0.148	0.192	0.024	1.480	1.924	0.236
8	0.023	0.019	0.255	0.006	0.107	1.390	0.032

2.3. Comparison of the activity of glucose oxidase preparations

Different enzyme preparations can be compared only in reaction mixtures having the same glucose oxidase activities. The previously mentioned preparations were compared after adjusting the glucose oxidase activities of the reaction mixtures to 0.26 and to 2.60 SU ml^{-1}, resp. The activities of these preparations (as determined by us), the ratio of their catalase and glucose oxidase activities, the parameters of enzyme addition are given in Table 3. The amount of gluconic acid formed from glucose by various glucose oxidase preparations, was plotted against reaction time in Fig. 6.

It is obvious from the figure that, at the 0.26 SU ml^{-1} concentration level, the rate of the reaction catalysed by the NBC preparation was the lowest. Thereafter followed, in increasing order, the unpurified preparations KÉKI Nos. 3 and 8 and the purified preparation (KÉKI GT). The highest reaction rate of glucose oxidation was exhibited by the SIGMA and MERCK preparations. When glucose oxidase activities of the reaction mixtures were increased 10-fold, the reaction rate of the NBC preparation was the lowest, then followed KÉKI No. 3, KÉKI No. 8 and the purified preparation (KÉKI GT). This sequence was reversed in the 180 to 240 min reaction time range. The reaction rate of the purified preparation decreased intensively with the progress of the reaction. A decrease in the reaction rate was also found in preparation KÉKI No. 8, but to a lesser extent than in the case of the purified preparation. Out of our preparations KÉKI No. 3 has shown the highest glucose oxidation rate, but significantly lower than the rates of the reaction catalyzed by the MERCK and SIGMA preparations. The reactions catalyzed by the two concentrations of the above mentioned preparations, the extent

1

oxidase, the enzyme concentration of reaction mixtures of various preparations and the reaction glucose oxidation

No. **3** 7190 SU g^{-1} and 900 BU g^{-1}; KÉKI No. **5** 1 300 SU g^{-1} and 160 BU g^{-1}; KÉKI of the reaction curves by regression analysis. (Tests at 3 concentration levels: I, II III)

glucose oxidase and mixture			Reaction rate			Increase in enzyme concentration		Increase in reaction rate	
III			I	II	III				
mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})	gluconic acid formation (μmole min^{-1})			II/I	III/I	II/I	III/I
0.551	2.775	0.27	0.94	7.50	11.00	7.59	14.12	7.98	11.70
0.413	2.969	0.37	1.64	5.69	11.88	3.89	10.87	3.47	7.24
2.760	3.588	0.44	0.62	6.95	11.36	10.00	18.65	11.21	18.32
0.214	2.782	0.06	1.60	8.41	10.96	5.63	11.26	5.26	6.85

Table

The effect of glucose oxidase concentration on reaction rate. The ratio of catalase to glucose
reaction rates of

Activities of preparations tested: SIGMA: 14 700 SU g^{-1} and 2 440 BU g^{-1}; MERCK:
rate was calculated from the linear part of the reaction curves by

Enzyme preparations		Concentrations of weighed-in enzyme preparations as well as and catalase in the reaction					
		I			II		
Name	ratio of catalase to glucose oxidase (R)	mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})	mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})
SIGMA	0.165	0.010	0.147	0.024	0.107	1.570	0.256
MERCK	0.283	0.019	0.286	0.081	0.148	2.230	0.633
NBC	0.080	0.115	0.172	0.013	1.070	1.600	0.128

Table

Effect of a ten-fold increase in glucose

The activities of preparations, the ratio of catalase and glucose oxidase activity, enzyme
rates were calculated from the linear part of the reaction curves by

Glucose oxidase preparations			Ratio of catalase to glucose oxidase (R)	Concentrations of weighed-in enzyme catalase in the		
	concentrations			I		
Name	glucose oxidase (SU g^{-1})	catalase (BU g^{-1})		mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})
SIGMA	14 700	2 440	0.165	0.017	0.258	0.001
MERCK	15 096	4 280	0.283	0.017	0.260	0.0025
NBC	1 500	120	0.080	0.172	0.259	0.0007
KÉKI No. 8	13 000	300	0.023	0.021	0.259	0.0002
KÉKI No. 3	7 190	900	0.125	0.036	0.260	0.0011
KÉKI GT	37 200	—	—	0.007	0.255	—

of increase in the reaction rate as a consequence of the increase in enzyme activities of the reaction mixtures, are given also in Table 3.

As can be seen in Fig. 6, the glucose oxidation rates, catalyzed by various preparations when the glucose oxidase activity of the reaction mixtures was adjusted to the same level, were different. The reaction rates show an increasing tendency with the increase of the specific activity of the preparations, and with the increase of the ratio of their catalase to glucose oxidase activity (KÉKI No. 5 and NBC preparations; as well as KÉKI No. 3 and No. 8 preparations).

2

oxidase, the enzyme concentration of reaction mixtures of various preparations and the their glucose oxidation

15 096 SU g^{-1} and 4 280 BU g^{-1}; NBC: 1 500 SU g^{-1} and 120 BU g^{-1}. Reaction regression analysis. (Tests at 3 concentration levels: I, II and III)

of glucose oxidase mixture			Reaction rate			Increase in enzyme concentration		Increase in reaction rate	
III			I	II	III				
mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})	gluconic acid formation (μmole min^{-1})			II/I	III/I	II/I	III/
0.214	3.146	0.522	2.66	14.00	17.10	10.70	21.40	5.26	6.43
0.296	4.466	1.266	4.56	16.55	21.30	7.79	15.58	3.63	4.67
1.930	2.890	0.232	0.76	4.86	6.94	9.30	16.78	6.39	9.13

3

oxidase concentration on the reaction rate

concentration of reaction mixtures and the reaction rate of glucose oxidation. Reaction regression analysis. (Tests at 2 concentration levels: I and II)

preparations as well as of glucose oxidase and reaction mixture			Reaction rate		Increase in enzyme concentration	Increase in reaction rate
II			I	II		
mg ml^{-1}	GO (SU ml^{-1})	catalase (BU ml^{-1})	gluconic acid formation (μmole min^{-1})		II/I	II/I
0.175	2.58	0.0141	4.02	14.33	10	3.56
0.172	2.60	0.0251	4.38	14.63	10	3.34
1.720	2.59	0.0071	1.13	4.57	10	4.04
0.210	2.59	0.0022	1.78	9.30	10	5.22
0.360	2.60	0.0011	1.53	10.00	10	6.53
0.070	2.55	—	1.78	9.29	10	5.22

2.4. The effect of the ratio of catalase to glucose oxidase activity on the glucose oxidation rate

The reaction rates catalyzed by various glucose oxidase preparations, in reaction mixtures adjusted to the same GO level, were plotted against the ratio of catalase and glucose oxidase activities (Fig. 7).

Fig. 7 shows that, in reaction mixtures of the same glucose oxidase activity (E_G), the reaction rates increased with the increase in the ratio of catalase and glucose oxidase activities of the preparations (NBC, KÉKI No. 3, SIGMA,

MERCK). Preparations having high glucose oxidase activities and zero or very low ratio of catalase and glucose oxidase activities (KÉKI GT: 37 000 SU g^{-1} and KÉKI No. 8: 13 000 SU g^{-1}) can not be compared with preparations of lower glucose oxidase activity and of higher catalase : glucose oxidase ratio.

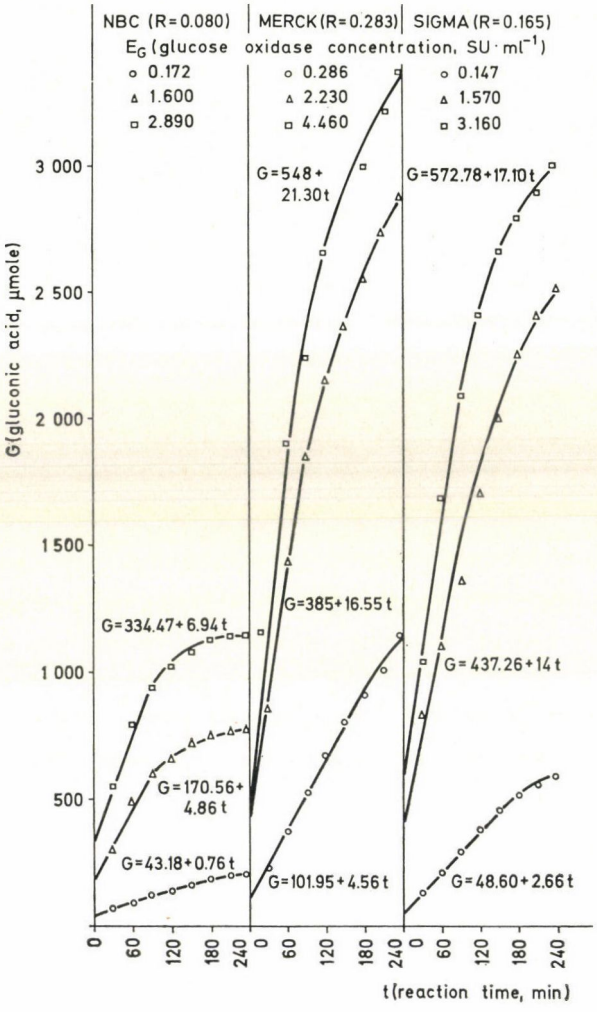

Fig. 4. Effect of enzyme concentration (E_G) on rate of glucose oxidation by some foreign commercial glucose oxidase preparations with different R values. (The activities of the enzymes and the reaction mixtures are given in Table 2. Each experimental point represents the mathematical mean of three parallels. Equations were calculated by regression analysis from the linear part of the curves.)

2.5. The effect of catalase on gluconic acid formation

In the course of our work it was found that the ratio of catalase to glucose oxidase might exert some, even if not unequivocal influence, on the reaction rate of glucose conversion.

Catalase preparations of various origin were tested for the role of catalase in the glucose-oxidase-catalyzed reaction. Catalase preparations of liver, bacterial and fungal origin were added at two concentration levels to the reac-

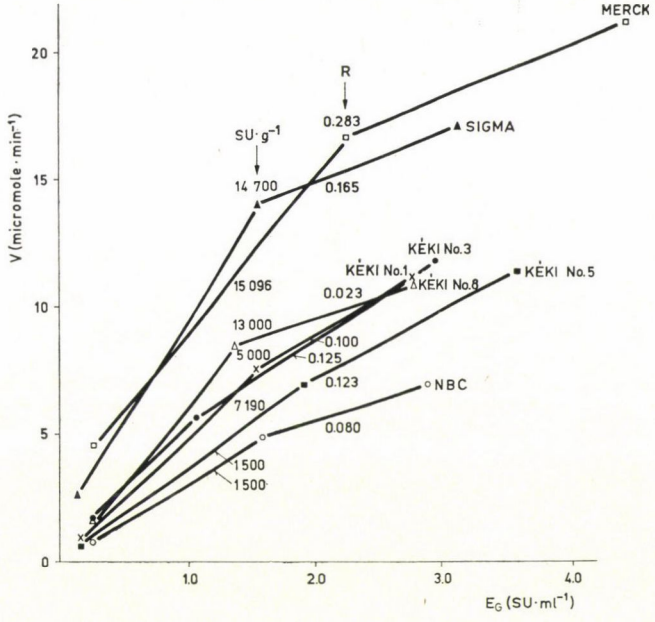

Fig. 5. Effect of enzyme concentration (E_G) on the reaction rate (V) of glucose oxidation by various Hungarian (KÉKI) and foreign glucose oxidase preparations having different R values

tion mixtures of the glucose oxidase preparation KÉKI No. 8. After the addition of glucose oxidase and catalase preparations the volume of the reaction mixture was adjusted to 29 ml with distilled water. Reaction mixtures containing only glucose oxidase and substrate served as the control. Samples were taken at 30-min intervals during a 4-hour period and gluconic acid conversion was determined by the kinetic titrimetric method (see para. 1.1.). The activities of the enzymes as well as the R value of the reaction mixtures are given in Table 4, while gluconic acid formation was plotted against the incubation time in Fig. 8. Reaction rates calculated by regression analysis from the linear part of the curves are given in Table 4.

As can be seen in the figure, gluconic acid formation was not linear throughout the whole incubation period, even if the reaction mixtures contained various kinds of catalases. The addition of catalases resulted in a certain increase in the reaction rate in the linear phase and a more significant increase in the declining phase. The highest increase in reaction velocity was obtained

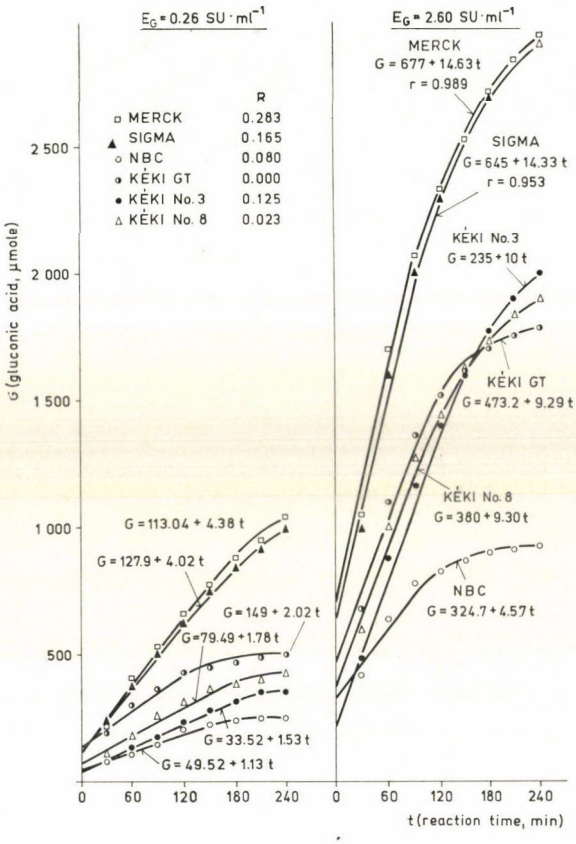

Fig. 6. Effect of two GO concentrations (E_G) on the reaction rate of glucose oxidation by various Hungarian (KÉKI) and foreign preparations. The activities of the enzymes and of the reaction mixtures are given in Table 3. (Each experimental point represents the mathematical mean of three parallels. Equations were calculated by regression analysis from the linear part of the curves.)

with the use of *Penicillium* catalase. The 7.5- and 14.0-fold increase in the R value by *Penicillium* catalase resulted in a 2.95- and 3.61-fold increase in the reaction rate, resp. The increase of the R value to about the same extent caused by addition of liver and bacterial catalase resulted only in a 1.22—1.39- and 1.35—1.38-fold increase in the reaction rate.

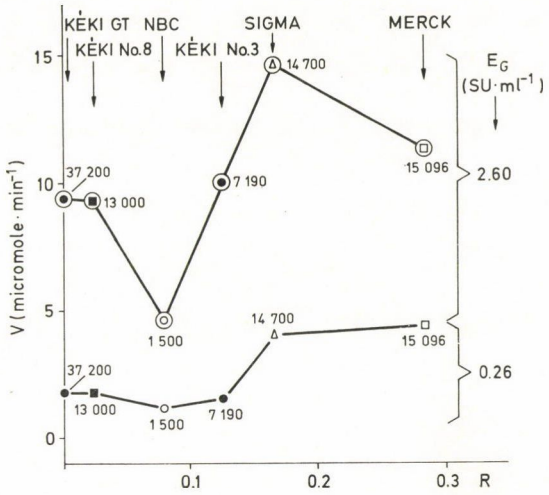

Fig. 7. Dependence of reaction rate (V) on catalase: GO ratio (R) of glucose oxidase preparations of varying GO content. (Numbers at experimental points indicate GO content of preparations in SU g^{-1}. Two levels of GO concentration [E_G] were applied in the reaction mixtures.)

Table 4

Effect of the addition of catalase preparations of various origin on gluconic acid formation by glucose oxidase preparation KÉKI No. 8

(Glucose oxidase activity: 13 000 SU g^{-1}; concentration of GO preparation in the reaction mixture: 0.019 mg ml^{-1})

Catalase preparations used		Reaction mixture				Reaction rate (gluconic acid formation, μmole min^{-1})	Increase* caused by the catalase preparation in	
Name	Concentration (BU g^{-1})	catalase added (BU ml^{-1})	actual enzyme activities		ratio of catalase to glucose oxidase (R)		R value	reaction rate
			GO (SU ml^{-1})	catalase (BU ml^{-1})				
Liver catalase (SIGMA C-10)	12 000	0.0372	0.247	0.0429	0.1736	2.17	7.54	1.22
		0.0744		0.0801	0.3240	2.48	14.08	1.39
Bacterial catalase (MERCK)	8 000	0.0372	0.247	0.0429	0.1736	2.40	7.54	1.35
		0.0744		0.0801	0.3240	2.46	14.08	1.38
Penicillium catalase (Ukr.N.I.I.S.L.P., Kiev)	10 000	0.0358	0.247	0.0415	0.1680	5.25	7.30	2.95
		0.0717		0.0774	0.3130	6.43	13.61	3.61
Test glucose oxidase KÉKI No. 8	300	—	0.247	0.0057	0.023	1.78	—	—

* Figures indicate the ratio of values obtained with and without added catalase

The effect of the addition of catalases on the reaction rate was also examined with the NBC glucose oxidase preparation. The enzyme activities of the preparations as well as the enzyme activities and the catalase-to-glucose oxidase ratio of the reaction mixtures are summarized in Table 5. The conditions of incubation and the determination of glucose conversion were the same as described above. The gluconic acid concentrations of the reaction mixture

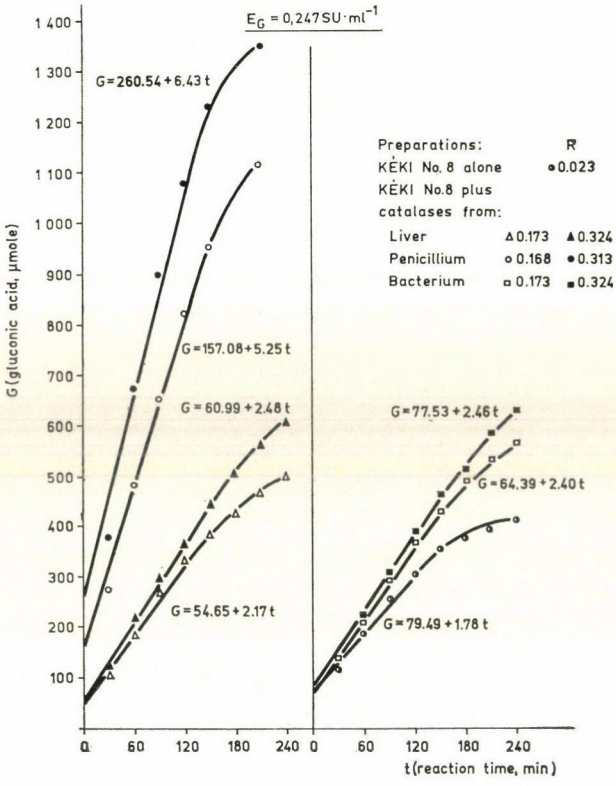

Fig. 8. Effect of addition of catalase preparations on the glucose oxidation rate of glucose oxidase preparation KÉKI No. 8 at various values of R in the reaction mixture. (The activities of the enzymes and the reaction mixtures are given in Table 4. Each experimental point represents the mathematical mean of three parallels. Equations were calculated by regression analysis from the linear part of the curves.)

of the control (NBC) and in those containing two concentrations of catalase of various origin, were plotted against the reaction time in Fig. 9. Reaction rates calculated by regression analysis from the data of the linear parts of the curves are summarized in Table 5.

As can be seen in Fig. 9, the reaction rate of the control preparation decreased significantly after a 120-minute incubation period. The addition of

catalase resulted in linear phases 30—60 minutes longer and a lower decrease in the reaction rates at the end of the incubation. About two- or four-fold increase in the R value of the NBC glucose oxidase preparation resulted in a 3.99—7.01-fold increase in reaction rates and 1.57—2.01-fold in the case of catalases of *Penicillium* and *Aspergillus* origin, while 1.10—1.32-fold and 1.11—1.26-fold only in the case of catalases from liver and bacteria.

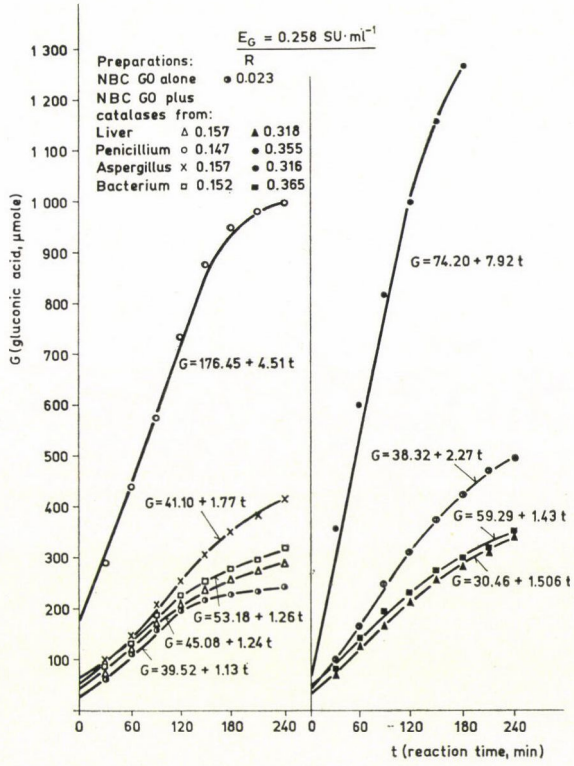

Fig. 9. Effect of addition of catalase preparations on the glucose oxidation rate of the NBC glucose oxidase preparation at various values of R in the reaction mixtures. (The activities of enzymes and reaction mixtures are given in Table 5. Each experimental point represents the mathematical mean of three parallels. Equations were calculated by regression analysis from the linear part of the curves.)

2.6. H_2O_2 inhibition

The fact that the reaction rate increased when catalase preparations were added to the reaction mixture indicated the possibility of H_2O_2 inhibition.

A glucose oxidase preparation (KÉKI No. 72/9) having a catalase : glucose oxidase ratio of 0.218 was used for studying the effect of H_2O_2 and

catalase on the reaction catalyzed by glucose oxidase. The reaction mixtures contained 25 ml substrate (1, 0.5, 0.2, 0.1, 0.05 and 0.025 *M* glucose, resp. dissolved in acetate buffer, pH = 5.1) + 1 ml of a 1 mg ml^{-1} solution of GO preparation KÉKI No. 72/9 + 3 ml of distilled water. These samples served as a control. In the second group the 3-ml portions of distilled water were replaced by 2.5 ml distilled water and 0.5 ml of H_2O_2. The H_2O_2 molarity of the reaction mixture was 0.17 *M*. The third group contained, in

Table 5

Effect of the addition of catalase preparations of various origin on gluconic acid formation by NBC glucose oxidase preparation

(Glucose oxidase activity: 1 500 SU g^{-1}; concentration of GO preparation in the reaction mixture: 0.115 mg ml^{-1})

Catalase preparations used		Reaction mixture				Reaction rate (gluconic acid formation, μmole min^{-1})	Increase* caused by the catalase preparation in	
Name	Activity (BU g^{-1})	catalase added (BU ml^{-1})	actual enzyme activities		ratio of catalase to glucose oxidase (R)		R value	reaction rate
			GO (SU ml^{-1})	catalase (BU ml^{-1})				
Liver catalase (SIGMA C-10)	12 000	0.0206	0.258	0.0406	0.1573	1.24	1.97	1.10
		0.0620		0.0820	0.3178	1.506	3.97	1.33
Bacterial catalase (MERCK)	8 000	0.0193	0.258	0.0393	0.1520	1.26	1.90	1.11
		0.0744		0.0944	0.3658	1.43	4.57	1.26
Penicillium catalase (Ukr.N.I.I.S.L.P., Kiev)	10 000	0.0179	0.258	0.0379	0.1470	4.51	1.84	3.99
		0.0717		0.0917	0.3550	7.92	4.43	7.01
Aspergillus catalase (U.V.U.P.P., Prague)	—	0.0206	0.258	0.0406	0.1573	1.77	1.97	1.57
		0.0616		0.0816	0.3160	2.27	3.95	2.01
Test glucose oxidase NBC	—	—	0.258	0.0200	0.0800	1.13	—	—

* Figures indicate the ratio of values obtained with and without added catalase

addition to substrate, glucose oxidase and H_2O_2, 1 ml of catalase solution (MERCK bacterial catalase) and 1.5 ml of distilled water.

The glucose oxidase and catalase concentration as well as the ratio of catalase to glucose oxidase of the reaction mixtures are given in Table 6.

The conditions of incubation and determination of gluconic acid formation were identical with those described in para. 1.1.

The reaction rates calculated in the case of each substrate concentration by regression analysis, containing H_2O_2 and catalase resp., are also given in Table 6.

Table 6

Effect of H_2O_2 and catalase addition on the reaction velocity of glucose oxidation at various substrate concentrations

Enzyme preparations: *Glucose oxidase* KÉKI No. 72/9: 6 400 SU g^{-1}; *Bacterial catalase* (MERCK): 1 400 BU g^{-1}. Enzyme concentrations in the reaction mixture: 0.228 SU ml^{-1}; 0.050 BU ml^{-1} (Groups I and II) and 0.095 BU ml^{-1} (Group III)

Glucose concentration (*M*)	Reaction rate (μmole min^{-1})				
	Group I	Group II		Group III	
	glucose oxidase (R = 0.218)	glucose oxidase + H_2O_2 (0.17 *M*) (R = 0.218)	percentage of that of Group I (%)	glucose oxidase + H_2O_2 (0.17 *M*) + catalase (0.046 BU ml^{-1}) (R = 0.421)	percentage of that of Group I (%)
1.000	1.6357	0.8785	53.71	1.4261	87.18
0.500	1.6023	0.8486	52.96	1.3423	83.77
0.200	1.3952	0.7809	56.00	1.3357	95.73
0.100	1.4071	0.7060	50.17	1.2071	85.78
0.050	1.0214	0.6619	64.80	0.9943	97.35
0.025	0.8047	0.5642	70.11	0.7976	99.11

As seen in the table, the addition of 0.17 *M* H_2O_2 decreased the reaction rate substantially.

The increase in the R value of the reaction mixture from 0.218 to 0.421 effected by addition of bacterial catalase, significantly decreased the inhibitory effect of the H_2O_2 at each substrate concentration.

The values of the *Michaelis* constant (K_m) and V_{max} are given in Table 7.

Table 7

Effect of H_2O_2 and catalase on the Michaelis constant

Glucose oxidase KÉKI No. 72/9: 6 400 SU g^{-1}; 1 400 BU g^{-1}

Groups tested	Enzyme concentrations in the reaction mixture		R	K_m	V_{max}
	glucose oxidase (SU ml^{-1})	catalase (BU ml^{-1})			
I. Glucose oxidase	0.228	0.050	0.218	0.0099	1.662
II. Glucose oxidase + H_2O_2 (0.17 *M*)	0.228	0.050	0.218	0.0189	0.835
III. Glucose oxidase + H_2O_2 (0.17 *M*) + catalase (0.046 BU ml^{-1})	0.228	0.096	0.421	0.0100	1.427

According to the data of Table 7, in the reaction mixture containing 0.17 *M* H_2O_2, the value of V_{max} decreased by 50 per cent with reference to that containing glucose oxidase only. When 0.046 BU ml^{-1} catalase was added, along with H_2O_2, to the reaction mixture (Group III), the V_{max} value was 86 per cent of that of the control (Group I). The values of the *Michaelis* constants were equal (0.0099 and 0.0100) in the control sample (Group I) and in that containing H_2O_2 and catalase (Group III), while in samples containing glucose oxidase and H_2O_2 (Group II) the K_m value was nearly twice that of the control group.

2.7. Inhibitory effect of δ-gluconolactone

The reaction products of the action of glucose oxidase are δ-gluconolactone and H_2O_2. Since H_2O_2 proved to be an inhibitor of the enzyme, the inhibitory effect of δ-gluconolactone was tested, too. Reaction mixtures of Group I (Table 8) consisted of 25 ml glucose substrate of various molarity, 1 ml glucose oxidase solution (KÉKI No. 72/10, conc.: 1 mg ml^{-1}) and 3 ml distilled water. Groups II and III contained 0.05 *M* and 0.10 *M* δ-gluconolactone, resp. in addition to the enzyme and substrate components, and the reaction mixtures were adjusted to 29 ml with distilled water. Substrates were prepared with acetate buffer of pH 5.1 in the case of Group I and pH 6.0 in the case of Group II and III to decrease the acidity caused by the hydrolysis of the inhibitor. In order to minimize the hydrolysis of the lactone it was added to the reaction mixtures just before the addition of the enzyme. The incubation of the reaction mixtures and the determination of glucose conversion were the same as given in para. 1.1. Samples were taken every 30 minutes for 3 hours.

The reaction rates (calculated by regression analysis) were given as a function of the substrate concentration, in all three groups tested, in Table 8.

The average rates of gluconic acid conversion in the reaction mixtures containing 0.05 and 0.1 *M* lactone (Groups II and III) were about 95 and 86 per cent of that of the control group (I) not containing lactone. The above data show that, with the doubling of the δ-gluconolactone concentration of the reaction mixture, the inhibition increases.

The values of the *Michaelis* constant (K_m) and V_{max} are given in Table 9.

It can be seen from the data of Table 9 that the addition of δ-gluconolactone (0.05 *M*) to the reaction mixtures resulted in V_{max} values 5 and 15% lower and in K_m values about 6 and 27% higher than that of Group I.

Table 8

Effect of δ-gluconolactone on reaction velocity of glucose oxidation at various substrate concentrations

Glucose oxidase preparation KÉKI No. 72/10: 5 800 SU g^{-1}; 1 400 BU g^{-1}. Enzyme concentration in the reaction mixture: 0.207 SU ml^{-1}; 0.050 BU ml^{-1} (R = 0.242)

Glucose concentration (*M*)	Reaction velocity (μmole min^{-1})				
	Group I	Group II		Group III	
	glucose oxidase	glucose oxidase + δ-gluconolactone (0.05 *M*)	Percentage of that of Group I (%)	glucose oxidase + δ-gluconolactone (0.10 *M*)	Percentage of that of Group I (%)
1.000	1.4842	1.4190	95.6	1.2476	84.0
0.500	1.4523	1.3261	91.3	1.2071	83.0
0.200	1.3261	1.2166	91.7	1.1833	89.23
0.100	1.1452	1.1119	97.1	0.9738	84.96
0.050	0.9595	0.9226	96.1	0.8142	85.0
0.025	0.7119	0.6761	95.0	0.6261	87.9

Table 9

Effect of δ-gluconolactone on values of the Michaelis constant (K_m) and V_{max}

Glucose oxidase KÉKI No. 72/10: 5 800 SU g^{-1}; 1 400 BU g^{-1}

Groups tested	Enzyme concentration of reaction mixture		R	K_m	V_{max}
	glucose oxidase (SU ml^{-1})	catalase (BU ml^{-1})			
I. Glucose oxidase	0.207	0.050	0.242	0.0122	1.466
II. Glucose oxidase + δ-gluconolactone (0.05 *M*)	0.207	0.050	0.242	0.0130	1.397
III. Glucose oxidase + δ-gluconolactone (0.10 *M*)	0.207	0.050	0.242	0.0155	1.254

3. Conclusions

The *Michaelis* constant determined for glucose oxidase preparations KÉKI Nos. 1, 72/9 and 72/10 proved to be 0.0186 and 0.0099 and 0.0122 *M*, resp. These values were higher than that (0.0042) found by KEILIN and HARTREE (1948) and lower than those obtained by FRANKE and LORENZ (1937) and by GIBSON *et al.* (1964) (K_m = 0.026 and 0.11, resp.) and similar to those reported for glucose oxidases of *Penicillium* origin (0.015 and 0.0095 *M*; SWOBODA & MASSEY, 1965).

There was an increasing tendency in the reaction rate with the increase in glucose oxidase activity of the preparations (Fig. 10) and in most cases with the increase in the ratio of their catalase and glucose oxidase activity.

When the velocity of gluconic acid formation was plotted against the square root of enzyme concentration, the relationship was found approximately linear (Fig. 11). Similar results were obtained when the logarithm of the reaction velocity was plotted as a function of the logarithm of the enzyme concentration (Fig. 12).

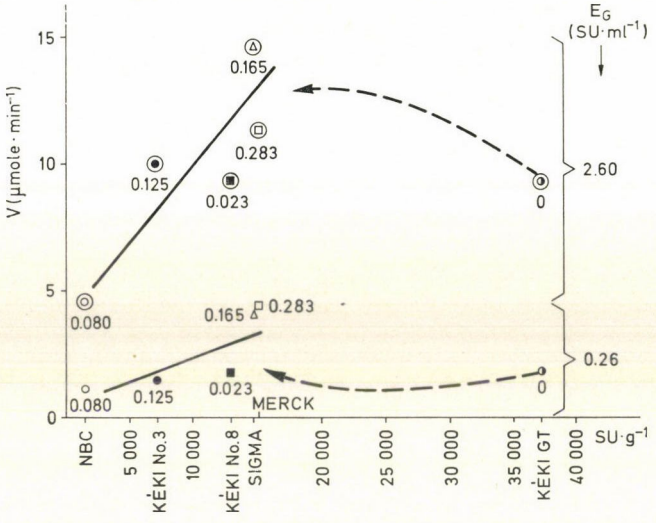

Fig. 10. Dependence of the reaction rate (V) on glucose oxidase content (SU g^{-1}) of enzyme preparations of varying catalase content used at two levels of GO concentration (E_G) in the reaction mixture. Numbers at the experimental points indicate the ratio of catalase to GO (R). (Data: Table 3)

For routine assays, when continuous titration cannot be done because of the large number of samples, enzyme concentration of the reaction mixture must be chosen so that the reaction rate should remain unchanged throughout the whole reaction time. The enzyme concentration of reaction mixtures must not be higher than 0.200 SU ml^{-1}.

The decrease in the reaction rate with the progress of the reaction time indicates enzyme inactivation.

The inactivation of the enzyme can be determined by graphical analysis, too (SELWYN, 1965) when the amounts of gluconic acid converted by different concentrations of the enzyme preparations are plotted against an abscissa showing reaction time multiplied by enzyme concentration (Fig. 13). In case there is no inactivation of the enzyme, gluconic acid values measured at various enzyme concentrations should fall on one curve. If the enzyme is denatured

during the reaction, the gluconic acid quantities for different concentrations of enzyme follow different curves.

According to Fig. 13, gluconic acid values for the various GO concentrations of the examined preparations usually followed different curves.

Studies on commercial GO preparations of various origin revealed that the rate of gluconic acid formation is strongly affected by a number of largely

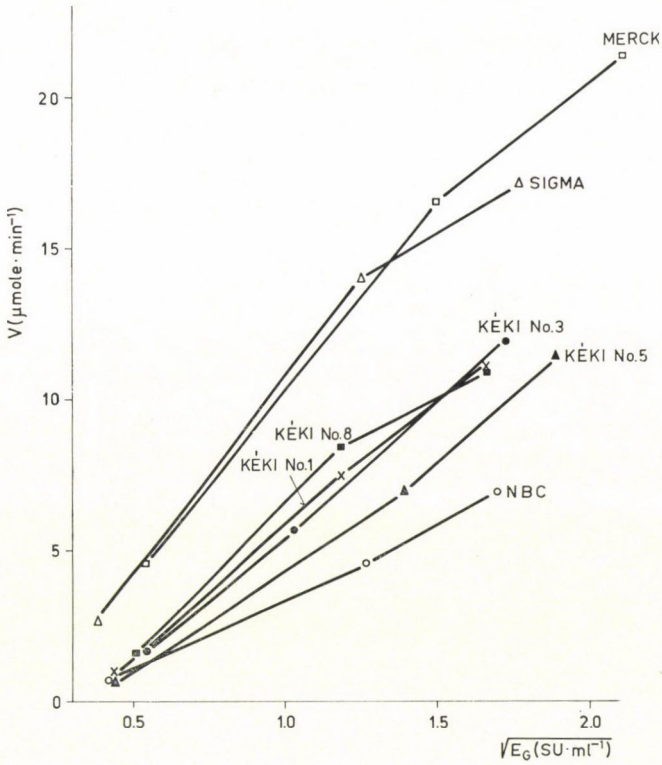

Fig. 11. Rate (V) of gluconic acid formation as a function of the square root of glucose oxidase concentration (E_G). (Tables 1 and 2)

unknown factors. As a result of this, it became apparent that techniques, widely used at present to measure GO activity, need urgent revision. Rates of the glucose → gluconic acid reaction, as measured with various commercial GO preparations, exhibited large differences in reaction mixtures containing the same number of *Sarrette* units (SU), the latter being the most widely accepted measure of GO activity. That is, correlation between the IUB-advocated kinetic units (as expressed in μmoles per minute) and the traditional SU values is poor.

The addition of catalases decreased the inactivation of glucose oxidase enzymes. Of the catalases from liver, bacteria and fungi the latter was the most effective in decreasing the inhibition. The 7—14-fold increase of the catalase : glucose oxidase ratio of the reaction mixtures by catalase preparations from liver, bacteria and *Penicillium* increased the velocity of the reaction catalyzed by glucose oxidase KÉKI No. 8 1.22—1.39-, 1.35—1.38- and 2.95—3.61-fold, resp.

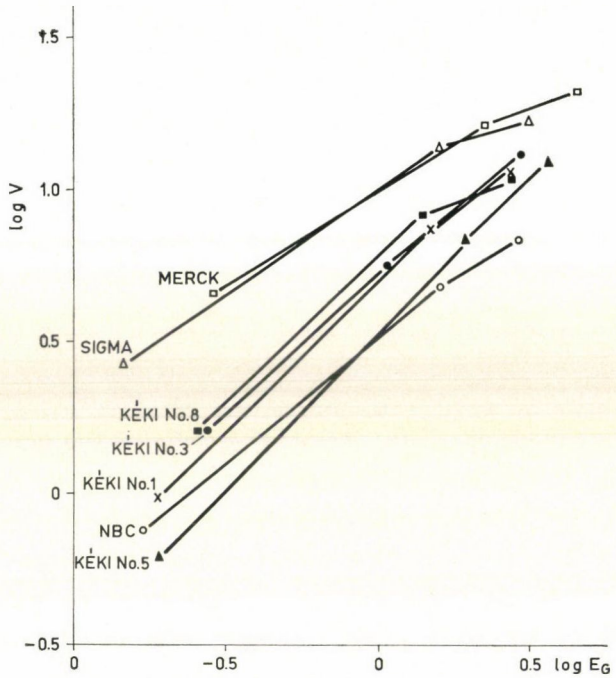

Fig. 12. Dependence of reaction rate (V, μmole min^{-1}) of gluconic acid formation on glucose oxidase concentration (E_G, SU ml^{-1}) with various enzyme preparations, in a double-logarithmic plot. (Data: Tables 1 and 2)

The 2.0- and 4.0-fold increase in the ratio of catalase to glucose oxidase of the NBC glucose oxidase preparation by the addition catalases from *Penicillium* and *Aspergillus* resulted in a 4.0—7.0-fold, and in a 1.6- and 2.0-fold increase in the reaction rate, respectively. When catalases of liver and bacterial origin were used the reaction rate increased only 1.1—1.4- and 1.1—1.26-fold as compared to that containing no catalase preparation.

On the basis of the above observations, inhibition by H_2O_2 was suggested. This was tested by the addition of 0.17 *M* H_2O_2 to the reaction mixture of a glucose oxidase enzyme when the reaction rate decreased by about 42%. But when the above concentration of H_2O_2 was added together with a bacteria

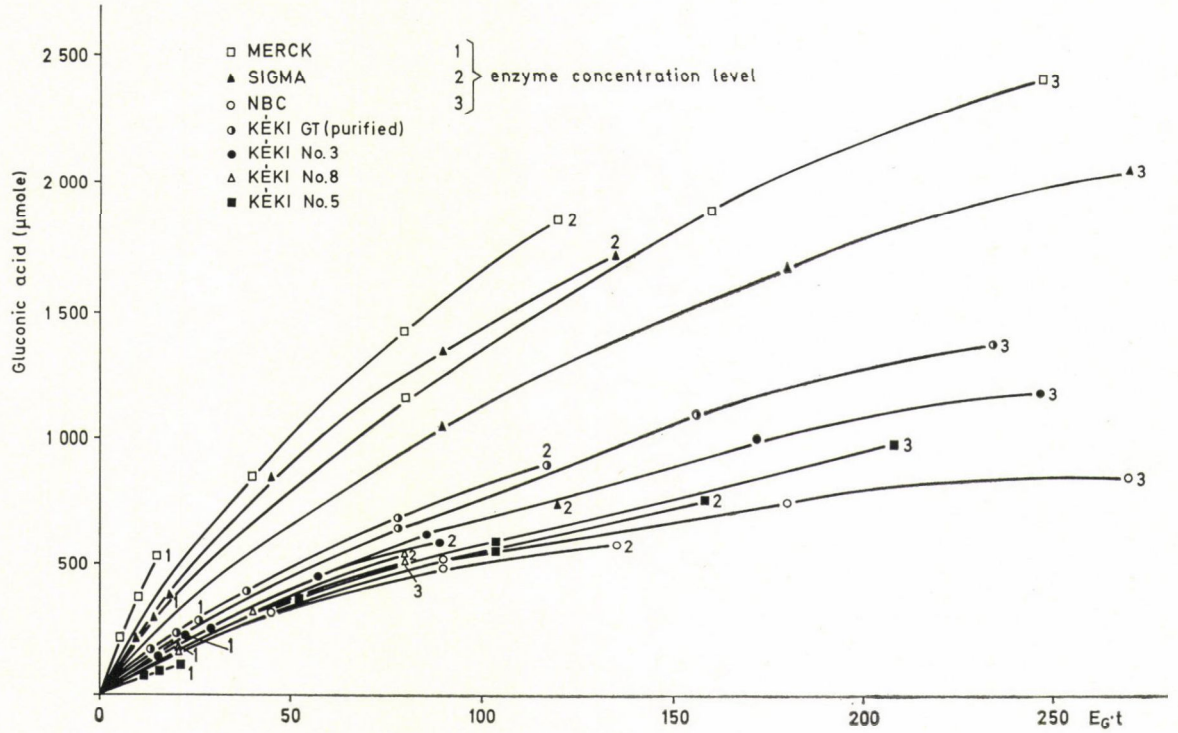

Fig. 13. Rates of gluconic acid formation of some foreign and Hungarian (KÉKI) glucose oxidase preparations plotted against incubation time [t (min) up to 180 min] multiplied by the enzyme concentration [E_G (SU ml^{-1})]

atalase preparation, the decrease of the reaction rate was only about 10% of hat co ntaining no H_2O_2.

KLEPPE (1966), studying the inactivation of glucose oxidase enzyme by H_2O_2, found that the reduced enzyme was inactivated more rapidly than the oxidized one. According to the spectra of the active and inactivated enzyme and to the results of amino acid analysis, the modification of methionine located near the active center was suggested. KLEPPE found that the K_m values of the H_2O_2-treated and the nontreated samples were the same, and it was only the V_{max} that had changed. Though he inactivated the enzyme prior to incubation and the reaction velocity was measured manometrically on the basis of O_2 uptake, our results concerning K_m values are not in agreement with the above statement.

The K_m value of the samples containing H_2O_2 was about the double of the control, containing only glucose oxidase (0.0189 and 0.0099 *M*, resp.), while in samples where H_2O_2 was added together with catalase the K_m value was equal to that of the control. The values of V_{max} calculated in samples containing H_2O_2 and H_2O_2 with catalase were about 50 and 86 per cent, resp., of that of the control. The 14 per cent lower V_{max} value might be the result of the inhibitory effect of the other reaction product, namely δ-gluconolactone.

The addition of δ-gluconolactone to the reaction mixture resulted in a slight inhibition of the action of glucose oxidase enzyme. NAKAMURA and OGURA (1962) also reported on the inhibitory effect of δ-gluconolactone and supposed the binding of lactone to the active center of the glucose oxidase enzyme.

According to the *Lineweaver-Burk* plots of our results, the inhibition types of both H_2O_2 and δ-gluconolactone seem to belong to the mixed type of inhibition (DIXON & WEBB, 1958).

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Acta Alimentaria, Vol. 4. (1), pp. 63—79 (1975)

COMPARISON OF MILK-CLOTTING ENZYME PREPARATIONS BASED ON FRACTIONATION BY ISOELECTRIC FOCUSING

PART I. — INVESTIGATION OF MILK-CLOTTING ENZYME PREPARATIONS OF *MUCOR PUSSILLUS* ORIGIN

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(Received October 19, 1973)

Experiments were carried out to fractionate milk-clotting enzyme preparations by isoelectric focusing and to determine the pI value of the various enzyme components present. The preparations were produced in surface and submerged cultures of a strain of the mould *Mucor pusillus* Lindt. The main results were as follows.

(a) The proteolytic component of the enzyme preparation *E31* produced by surface fermentation can be divided, by isoelectric focusing, into at least two (in a very narrow pH range into three) fractions with isoelectric points of pI 3.81, 3.83 and 4.01, resp. The milk-clotting component of the preparation constitutes the main fraction with pI values of 3.98 to 4.01 and a smaller fraction with pI 3.83; the lipase component has three main and one smaller fraction with pIs 3.81, 3.83, 4.01 and 3.64, resp. Thus the other two enzyme components can, to a certain extent, be separated from the milk-clotting enzyme by fractionation according to their isoelectric points.

(b) Investigation of the proteolytic activity of the enzyme preparation *E32* produced by submerged fermentation reveals one main and two substantially smaller fractions. The pI value of the main fraction is 8.81, those of the two smaller fractions 9.13 and 8.37, resp. The milk-clotting and lipase activities greatly deteriorated in the course of focusing and could be detected only in minimal quantities in the fractions with maximum proteolytic activity.

(c) In the Japanese enzyme preparation *Meito Rennet*, produced by surface fermentation, the proteolytic, milk-clotting and lipase components appeared essentially in a common main fraction with an isoelectric point of pI 4.00. In addition, the proteolytic component gave another smaller fraction which contained 4% of the overall activity and had an isoelectric point of 4.68.

(d) There is a substantial difference between the isoelectric points of the enzyme preparations *E31* and *E32* produced from the same mould strain, but by surface and submerged fermentation technology, resp. This difference is presumably due to their different compositions, as supported by the finding that the maximum value of the proteolytic activity of the preparation *E32* is not accompanied by the absorption maximum measured at 280 nm, in contrast to the behaviour of the preparation *E31*.

These results have furnished further data in support of our earlier observations (MORVAI, 1971; VÁMOS-VIGYÁZÓ & MORVAI-RÁCZ, 1970; VÁMOS-VIGYÁZÓ *et al.*, 1973), namely that enzyme preparations produced by different fermentation technologies will have different composition.

In recent years we have prepared milk-clotting enzyme preparations by surface and submerged fermentation using strains of the mould *Mucor pusillus* Lindt and *Endothia parasitica* on a laboratory scale. The various properties of these enzyme preparations were studied and compared to the corresponding data of enzyme preparations produced abroad. Our work included the fractionation of the components of the various enzyme preparations according to

their isoelectric points and the determination of the isoelectric points of the milk-clotting, proteolytic and lipase components.

These investigations were carried out by means of the method of isoelectric focusing which, in recent years, has come to be used more and more widely all over the world. This method is essentially an electrophoresis performed in a pH gradient. This latter is produced in the appropriate pH range by means of the addition of Ampholine, an organic N-containing polymer (VESTERBERG & SVENSSON, 1966). Mixing of the separated protein fractions and an undue broadening of the already formed zones are prevented by the density gradient or by the simultaneous application of some other inert materials *e.g.*, of diverse gels. Accordingly, we distinguish between

— gel electrofocusing (WRIGLEY, 1968; GAINER, 1973; DELINCÉE & RADOLA, 1970)
— zone-convection electrofocusing (VALMET, 1969)
— and isoelectric focusing in the density gradient.

Of the three methods, isoelectric focusing in the density gradient is the most widely applied (HAGLUND, 1967; VESTERBERG & BERGGREN, 1967; LUNDBLAD, *et al.*, 1968; VESTERBERG *et al.*, 1967; TIRUNARAYANAN *et al.*, 1969; WADSTRÖM, 1967). This method includes separation on the column on which the density gradient is formed — depending on the nature of the sample — by the addition of saccharose (VESTERBERG *et al.*, 1967), ethyleneglycol (VESTERBERG, 1971a) or glycerine (VESTERBERG & BERGGREN, 1967).

Earlier we have applied the density gradient technique (POZSÁR-HAJNAL, 1972) to the fractionation of a crude lipase preparation produced by a *Pseudomonas* strain. In the present paper we wish to report on the fractionation by means of isoelectric focusing of enzyme preparations produced in our Institute by surface and by submerged fermentation, resp., of a strain of the mould *Mucor pusillus* Lindt and of a Japanese preparation from surface culture.

1. Materials and methods

1.1. Enzyme preparations

The milk-clotting enzyme preparation produced at the *Central Food Research Institute*, Budapest, by surface fermentation (POZSÁR-HAJNAL *et al.*, 1974a) will be designated by *E31*, and that produced by submerged fermentation (POZSÁR-HAJNAL *et al.*, 1974b) by *E32*.

The solutions of these enzyme preparations were dialyzed in a refrigerator against distilled water for 24 hours and then lyophilized.

Meito Rennet (*Takasago Perfumery Co.*, Tokyo, Japan) is an enzyme preparation produced by the surface fermentation of the strain *Mucor pusillus* Lindt, and will be designated by *MR*.

1.2. Isoelectric focusing

Isoelectric focusing was performed with the aid of the apparatus LKB 8100 (*LKB-Producter*, Stockholm). The column of 110 ml was cooled with running tap water. The density gradient was developed by means of a 50% solution of saccharose in water by means of a linear gradient mixing vessel. The pH gradient in the desired pH range was achieved by the addition of 1% of Ampholine; for a double run 2% of Ampholine was used. Ampholine, the sample under investigation and the density gradient solution were applied simultaneously to the column. The volume of the sample was 50 ml, its protein concentration 0.6 to 0.8 mg ml^{-1}, as determined by measuring its optical density (O.D.) at 260 and 280 nm and as calculated by means of the formula of COLOWICK and KAPLAN (1957). A 0.2% solution of ethanolamine served for the protection of the cathode, the anode was protected with a 0.1% solution of phosphoric acid. The thick electrode solution, in addition, contained 50% of saccharose. Depending on the pH range, the initial voltage was 340, 420 or 500 V; one run lasted from 48 to 72 hours.

After elution of the column 2.0 or 2 5 ml fractions were collected and their pH (*Radiometer*, Copenhagen) at 20°C, optical density at 260 and 280 nm (*Gilford* 2400 spectrophotometer), further their milk-clotting and proteolytic activities were measured. The lipase content in the fractions was determined by a semi-quantitative method.

1.3. Separation of Ampholine on Sephadex G-25

In the experiments of a preparative nature with *E32*, a Sephadex G-25 column was used for the separation of Ampholine. The diameter of the column was 2 cm, its height 12 cm, the volume of the sample was 3 ml. Together with the fractions collected after focusing, Dextran Blue 2000 was also applied to the column, which was eluted with distilled water at a rate of 1.5 ml per minute. Three-ml fractions were collected and their O.D. measured at 280 nm. In the fractions of maximum absorbance, the proteolytic and milk-clotting activities were also determined.

1.4. Determination of enzyme activities

Proteolytic activity of the solution used for focusing, *i.e.*, of the obtained fractions, was studied on casein substrate at pH 6.0. The O.D. of the trichloroacetic acid (TCA) soluble decomposition product obtained with the enzyme from the substrate after incubation for one hour at 35 °C, was measured at 280 nm against a standard solution to which the enzyme solution was added after precipitation of the substrate with TCA. A detailed description of the

method has been published elsewhere (VÁMOS-VIGYÁZÓ *et al.*, 1969). The absorbance at 280 nm of the TCA soluble decomposition product obtained under the above conditions from 1 mg of the enzyme preparation, *i.e.*, from 0.2 ml of focused solution, was taken as the index of enzyme concentration.

Milk-clotting activity was determined by means of the modified *Soxhlet* method (KETTING, 1955) and the enzyme concentration expressed in SU ml^{-1}. Five millilitres of 10 % rehydrated skimmed milk powder were used as substrate without $CaCl_2$ supplementation. The time of clotting (in sec) was measured

Table 1

Protein content, milk-clotting enzyme, proteolytic enzyme and lipase concentrations of the investigated enzyme preparations of Mucor origin

Symbol of enzyme preparations	Protein content, mg g^{-1}			Milk-clotting enzyme concentration, SU g^{-1}			Proteolytic enzyme concentration, U g^{-1}			Lipase concentration, U g^{-1}		
	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s
E31	3	702	30.0	3	48 668	25 096	3	1 050	240	4	11.0	2.0
										2	168*	
E32	3	511	5.1	3	139 200	30 000	3	3 690	1 120	4	41.5	1.3
MR	3	513	10.5	3	46 522	22 089	3	500	60	2	4.8*	

N = number of measurements
$\bar{x}$ = arithmetical average of the experimental results
s = standard deviation
* = data from MORVAI (1971) used for purposes of comparison. (Substrate: olive oil emulgated with lecithine. Activity is measured by the number of μmoles of fatty acid liberated in one hour.)

after the addition of 0.5 ml of the fraction in the desired dilution. The quantity of enzyme necessary for the clotting of 1 ml of milk at 35° C in 40 minutes equals 1 *Soxhlet* unit (SU).

In the enzyme preparations *E31* and *E32* subjected to fractionation, the lipase activity was determined on a substrate, containing 3 % of tributyrin stabilized with a 5 % gum arabic solution. The quantity of liberated butyric acid was determined by titration with NaOH to a constant pH value of 8.0 at 30 °C as a function of reaction time. In the first 5 minutes a linear relationship was obtained without exception, so that from the initial reaction period the value of activity was determined and expressed in μmoles of butyric acid. Activity was measured with the pH-stat of *Radiometer*, Copenhagen.

Lipase was semi-quantitatively determined after isoelectric focusing on 5-mm thick plates containing 0.15 % of tributyrin and 15 % of agar-agar by means of the agar-diffusion method. Holes of 7 mm diameters were made into the plates and enzyme activity was expressed as the diameter of the hydro-

lysis zone obtained with 0.05 ml of the fractions incubated at room temperature for one to three days.

The protein content of the enzyme solution used for focusing and of the fractions obtained after focusing was determined from the O.D. values measured at 260 and 280 nm and calculated by means of the formula of COLOWICK and KAPLAN (1957).

The protein content, milk-clotting, proteolytic enzyme and lipase concentrations in the investigated preparations are given in Table 1.

2. Results

First, the enzyme preparations were investigated in the pH gradient between 3 and 10 pH, then, depending on the appearance of enzyme activity, and in order to achieve better resolution, the run was repeated in the 3 to 6 or 7 to 10 pH range.

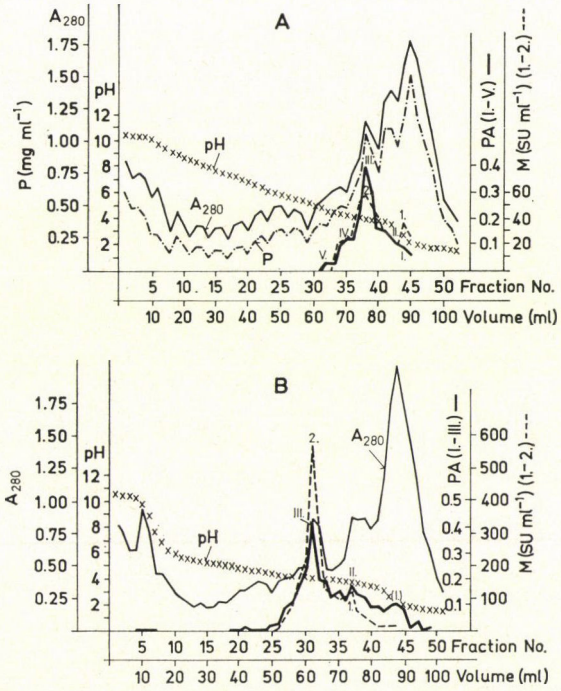

Fig. 1. Fractionation of the enzyme preparation *E31* according to the isoelectric point. A — 1% of Ampholine, pH 3 to 10; B — 1% of Ampholine, pH 3 to 6; P = protein content (mg ml^{-1}); A_{280} = absorption measured at 280 nm; PA = proteolytic activity (O.D. at 280 nm); M = milk-clotting activity (SU ml^{-1}). The Roman numerals represent the separating fractions of the proteolytic enzyme, the Arabic numerals those of the milk-clotting components. The numbers in brackets stand not for true fractions, but for protracted fraction components

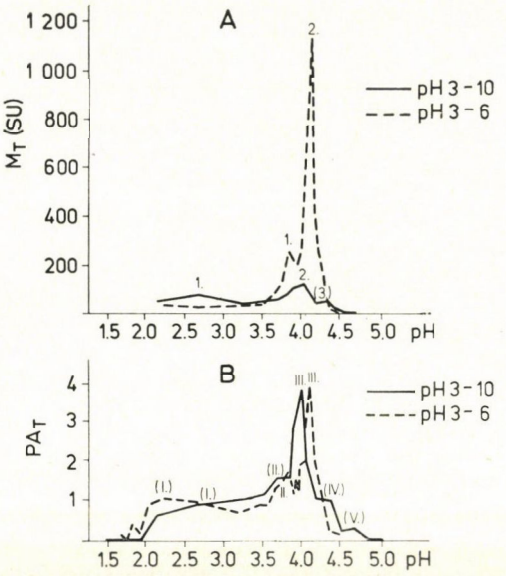

Fig. 2. Milk-clotting and proteolytic enzyme fractions of the enzyme preparation *E31 vs.* pH when run in two different pH ranges. M_T = total milk-clotting activity (SU); PA_T = total proteolytic activity. For other legend see Fig. 1

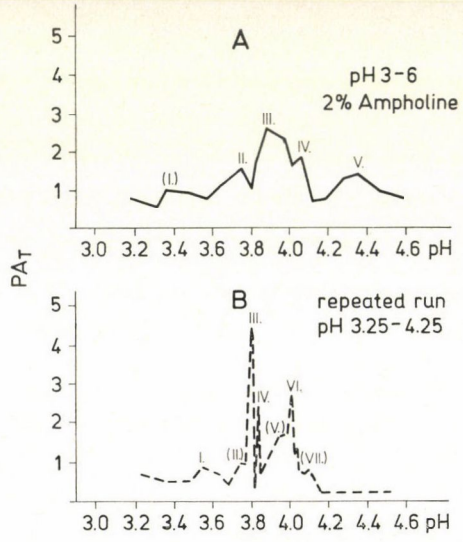

Fig. 3. Proteolytic fractions of the enzyme preparation *E31 vs.* pH. A — 2% of Ampholine, pH 3 to 6; B — repeated run after the collection of selected fractions. For legend see Fig. 2

2.1. Enzyme preparation E31

The results of focusing in case of the milk-clotting enzyme *E31* are shown in Fig. 1.

In part "A" of the figure, along with the O.D. of the fractions at 280 nm, the calculated protein content is also shown. Since the curve of the protein content fits well the absorbance curve measured at 280 nm, it failed to provide further information, so that in the next experiments these values had to be disregarded.

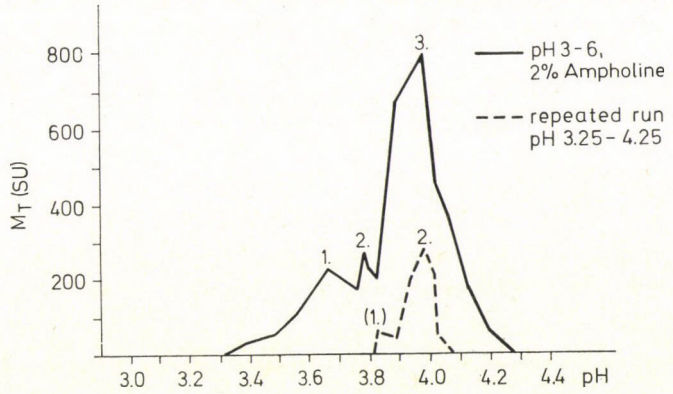

Fig. 4. Milk-clotting fractions of the enzyme preparation *E31 vs.* pH when run in two different pH ranges. For legend see Fig. 2

From the results of the experiments performed in the pH range between 3 and 10, it seemed that the major part of both the proteolytic and the milk-clotting enzyme activity belongs to the pH range between 3.70 and 4.17 (53 and 59%, resp., of the total activity, see Tables 2 and 3).

This is particularly conspicuous when the run is performed in a narrower pH range (Fig. 1 B) where both the proteolytic and the milk-clotting enzymes give two distinct fractions. This division is clearly visible in Fig. 2 in which the activities are plotted as a function of pH.

Comparison of the data in the tables and Fig. 2 shows that the two main components of the milk-clotting enzyme are situated within a pH range of 0.2. Nineteen per cent of the total milk-clotting activity has an isoelectric point of 3.83 and 62% a pI value of 4.11, while the pI of 23% of the proteolytic activity is 3.83 and within a pH range of 0.36, and 35% has a pI of 4.11 within a pH range of 0.17.

If the run is carried out within a very narrow pH range, separation will be even sharper, as shown in Figs. 3 and 4.

Table 2

Isoelectric points of the proteolytic fractions of enzyme preparations of Mucor origin and the distribution of fractions according to their pI values

Symbol of enzyme preparations Focusing conditions	Number of fraction	pI	pH range	Enzyme activity in % of the overall activity after focusing
E31	(I)*	—	1.92— 3.53	16
pH 3—10	(II)	—	3.53— 3.79	15
	III	3.99	3.79— 4.17	53
	(IV)	—	4.17— 4.51	14
	(V)	—	4.51— 4.98	2
E31	(I)	—	1.92— 3.19	13
pH 3—6	II	3.83	3.54— 3.90	23
	III	4.11	4.02— 4.19	35
E31	(I)	—	3.32— 3.57	8
pH 3—6	II	3.76	3.57— 3.81	14
2% Ampholine	III	3.89	3.81— 4.02	23
	IV	4.06	4.02— 4.12	9
	V	4.34	4.19— 4.58	14
E31	I	3.56	3.50— 3.69	7
pH 3.25—4.25	(II)	—	3.69— 3.78	5
(repeated run)	III	3.81	3.78— 3.82	16
	IV	3.83	3.82— 3.85	12
	(V)	—	3.85— 3.98	12
	VI	4.01	3.98— 4.02	15
	(VII)	—	4.02— 4.16	14
E32	I	9.34	8.64—10.28	79
pH 3—10	(II)	—	6.33— 8.64	13
E32	I	9.13	9.06— 9.20	7
pH 7—10	II	8.81	8.49— 8.98	58
	III	8.37	8.20— 8.49	12
	(IV)	—	7.98— 8.20	8
	(V)	—	6.50— 7.85	9
MR	I	1.89	1.69— 2.18	8
pH 3—10	(II)	—	2.18— 3.50	3
	III	4.04	3.86— 4.23	75
	(IV)	—	4.62— 5.03	3
	V	5.28	5.03— 5.52	1
MR	(I)	—	1.66— 2.96	13
pH 3—10	(II)	—	2.96— 3.76	17
(repetition)	III	3.98	3.86— 4.07	27
	(IV)	—	4.07— 4.44	20
	(V)	—	4.97— 5.39	3
MR	(I)	—	1.74— 3.29	2
pH 3—6	(II)	—	3.29— 3.80	22
	III	4.00	3.83— 4.11	51
	IV	4.68	4.55— 4.78	4

* The Roman numerals in the brackets do not represent true fractions, only protracted fraction components

Table 3

Isoelectric points of the milk-clotting fractions of the investigated enzyme preparations of Mucor origin and distribution of the fractions according to their pI values

Symbol of enzyme preparations ,Focusing conditions	Number of fraction	pI	pH range	Enzyme activity in % of the overall activity after focusing
E31	1	2.63	2.16 – 3.23	21
pH 3 – 10	2	3.99	3.70 – 4.17	59
	(3)*	—	4.17 – 4.51	14
E31	1	3.83	3.73 – 3.93	19
pH 3 – 6	2	4.11	3.97 – 4.19	62
E31	1	3.67	3.37 – 3.76	13
pH 3 – 6	2	3.79	3.76 – 3.83	18
2% Ampholine	3	3.98	3.83 – 4.12	64
E31	(1)	—	3.82 – 3.89	9
pH 3.25 – 4.25	2	3.98	3.89 – 4.02	78
repeated run				
E32	—	—	—	—
pH 3 – 10				
E32	1	8.73	8.62 – 8.81	minimal
pH 7 – 10				
MR	(1)	—	1.76 – 3.50	8
pH 3 – 10	(2)	—	3.50 – 3.86	8
	3	4.04	3.86 – 4.23	70
MR	1	3.98	3.76 – 4.31	90
pH 3 – 10				
(repetition)				
MR	(1)	—	1.91 – 3.62	7
pH 3 – 6	(2)	—	3.62 – 3.83	19
	3	4.00	3.85 – 4.07	66

* The meaning of the numerals in brackets is the same as in Table 2

In this experiment the Ampholine concentration was 2% (pH 3 to 6) in the first run, then the fractions containing the enzyme were collected and the run repeated without the addition of more Ampholine. In this case a linear pH gradient was obtained between pH 3.25 and 4.25 in 3/4 of the total volume of the column. Under these conditions it was possible to separate components, the isoelectric points of which differed only by a few hundredths, as can be seen in the figures. The pI values of the main components of proteolytic activity are 3.81, 3.83, and 4.01; that of the enzymes with milk-clotting activity 3.98,

thus it is possible to separate to a certain degree the two types of enzymes on the grounds of their isoelectric points. Similarly to the proteolytic enzyme, lipase, too, has three main fractions with pIs of 3.81, 3.83 and 4.01, and a maller fraction with anr isoelectric point of 3.64. Thus, under the given experimental conditions, the milk-clotting and lipase components can also be sepasated to some degree.

2.2. Enzyme preparation E32

Other tests have shown that the enzyme preparation *E32*, produced by submerged fermentation, has properties differing from those of the preparation *E31* (MORVAI, 1971; VÁMOS-VIGYÁZÓ & MORVAI-RÁCZ, 1970; VÁMOS-VIGYÁZÓ *et al.*, 1973). For this reason, the investigation of the isoelectric point of the enzyme appeared to be particularly promising.

The results of fractionation in the pH range between 3 and 10 are shown in Fig. 5 A. In this experiment the proteolytic component of the enzyme gave

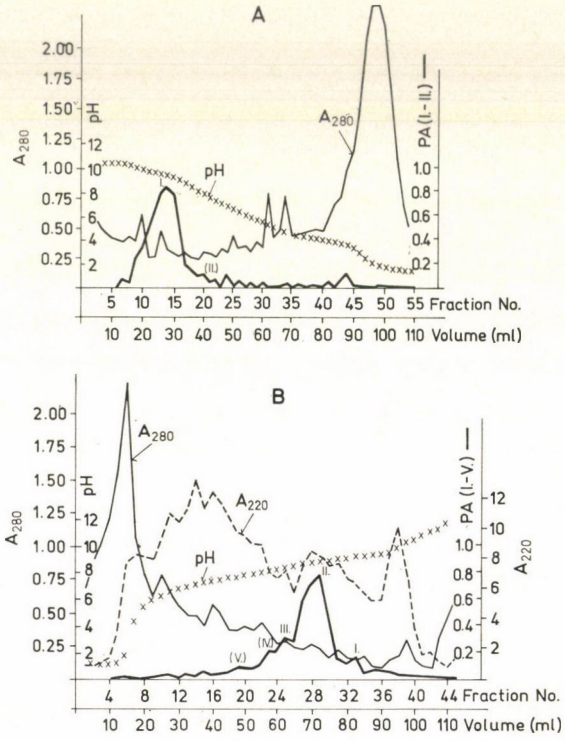

Fig. 5. Fractionation of the enzyme preparation *E32* according to the isoelectric point. A — 1% of Ampholine, pH 3 to 10; B — 1% of Ampholine, pH 7 to 10 and reversed pH gradient. A_{220} = absorption measured at 220 nm. For other legend see Fig. 1

essentially a single fraction, the maximum of which was at a pI value of 9.34, but, as appears from Fig. 6 and Table 2, the major part of total activity (79%) was situated in a wide pH range (within 1.64 pH units). The milk-clotting activity of the enzyme was so severly damaged by focusing that it could no longer be measured by the method hitherto applied (Table 3).

It was assumed that the milk-clotting activity of the enzyme preparation *E32* might not be stable near to its isoelectric point and therefore experiments were carried out to determine the milk-clotting activity of enzyme solutions, the pH values of which were adjusted in the range between 7.0 and 10.0 on a 0.5-increment when the enzyme was stored at room temperature for 1, 2 and 3 days, respectively. In this case the activity was also measured at pH 6.0. The results

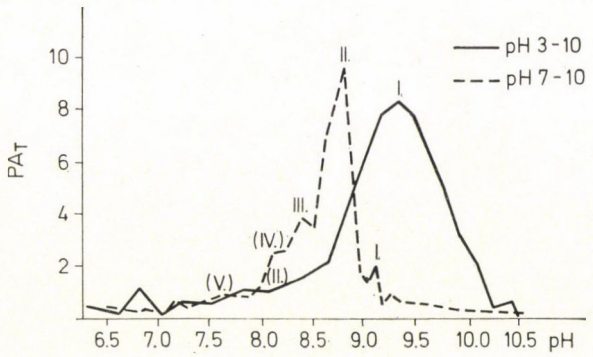

Fig. 6. Proteolytic fractions of the enzyme preparation *E32 vs.* pH when run in two different pH ranges. For legend see Fig. 2

indicate that up to pH 8.5, storage for 3 days causes a loss in activity of about 20%. Above pH 9, a loss of nearly the same extent appears already in the activity of freshly prepared enzyme solutions and at pH 9.0 a 70%, at pH 9.5 a 95% loss in activity is incurred after 3 days. It should be noted that not more than 18% of the proteolytic activity was recovered after focusing (Table 4). In our enzyme fractionations this was the only enzyme preparation which lost so much of activity during focusing.

Runs were also performed in narrower pH ranges (between 7 and 10), mainly in order to determine more accurately the value of the isoelectric point of the proteolytic component. In this experiment, bearing in mind the preparative aims, the poles of the current source were interchanged so as to obtain the enzyme at the lowest possible saccharose concentration and to facilitate thereby further separation. The results of focusing are shown in Fig. 5 B; proteolytic activity *vs.* pH was plotted in Fig. 6.

It appears from the figures that from the proteolytic component one main and two smaller fractions were obtained. In a pH range of 0.49, 58% of

Table 4

Enzyme activities recovered after isoelectric focusing of enzyme preparations of Mucor origin expressed as percentage of the overall applied activity

Symbol of enzyme preparations	Proteolytic activity (%)	Milk-clotting activity (%)
E31 pH 3—10	53	81
E31 pH 3—6	50	127
E31 pH 3—6 2% Ampholine	59	129
E31 pH 3.25—4.25 (repeated run)	105	43
E32 pH 3—10	18	0
E32 pH 7—10	24	minimal
MR pH 3—10	87	147
MR pH 3—10 (repetition)	55	190
MR pH 3—6	49	174

the total activity can be detected in the main fraction with a maximum at pI 8.81 (Table 2), thus the resolution of activity has been considerably higher than in the run in the pH range from 3 to 10, as can clearly be seen in Fig. 6. In this experiment, too, only a minimum milk-clotting activity was detected at pI 8.73 (Table 3), where a slight lipase activity was also shown to exist.

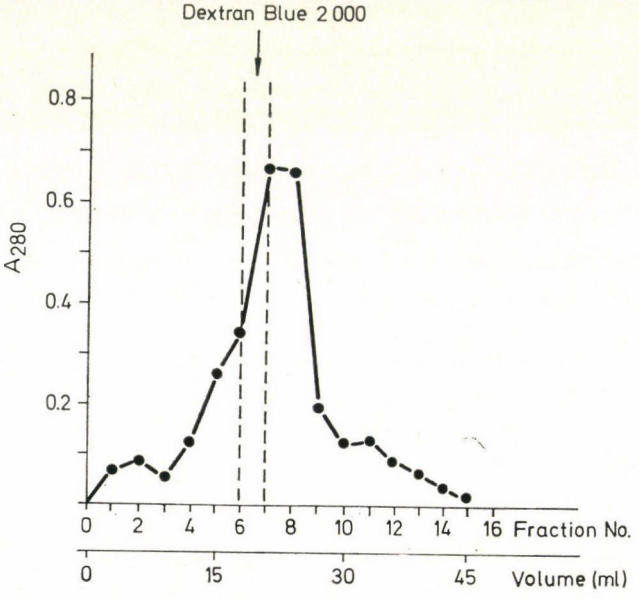

Fig. 7. Fractions of maximum proteolytic activity obtained by the focusing of the enzyme preparation *E32* with Ampholine between pH 7 to 10. Saccharose and Ampholine were removed on a Sephadex G-25 column. A_{280} = absorption measured at 280 nm

In this experiment a phenomenon was observed which did not seem to occur in the case of the other preparations. Proteolytic activity did not coincide with the absorption maximum at 280 nm, but showed monotonously decreasing O.D. values in the UV range from 220 nm upwards. This led us to the measurement of the absorbance of the fractions obtained by focusing also at 220 nm. It appears from Fig. 5 B that there is a lower extinction peak together with proteolytic activity at 220 nm, indicating that we are dealing here with an enzyme which is essentially different from the enzyme preparation *E31*.

The four fractions of maximum proteolytic activity were collected, and purified from saccharose and Ampholine on the Sephadex G-25 column (Fig. 7). It was attempted to recover the milk-clotting activity in this way.

However, the fractions with maximum absorption possessed only proteolytic activity, their milk-clotting component was hardly measurable. Thus, inactivation is an irreversible process.

2.3. The enzyme preparation Meito Rennet

For the sake of comparison, the isoelectric point of the enzyme preparation *Meito Rennet* was also determined. The proteolytic and milk-clotting activities *vs.* pH were plotted in Fig. 8.

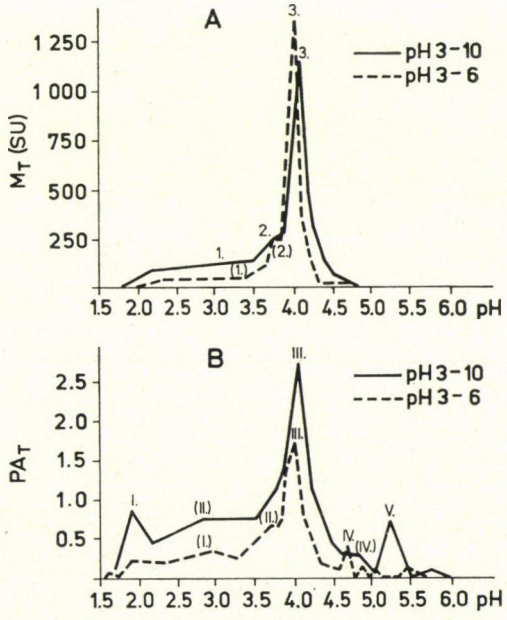

Fig. 8. Milk-clotting and proteolytic enzyme fractions of the enzyme preparation *MR vs.* pH when run in two different pH ranges. For legend see Fig. 2

The run in the pH range between 3 and 10 resulted in 3 fractions with proteolytic and in 1 fraction with milk-clotting activity. The main fraction of the proteolytic component (containing 75 % of the total activity) and the milk-clotting component were found at pI 4.04 in a 0.37 pH range. The other two fractions of the proteolytic component made up 8 % of total activity and were at pI 1.89 within a pH range of 0.49, while 1 % of total activity was measured at pI 5.28 in a pH range of 0.49. Lipase activity was detected between pH 2.0 and 4.8 with a maximum at pH 4.0.

When the experiment was performed in a narrower pH range, two proteolytic and 1 milk-clotting fractions were obtained from the enzyme. The isoelectric point of the main fraction was shifted to pI 4.0 and contained 52 % of the proteolytic activity in a pH range of 0.28, as well as 66 % of the milk-clotting activity within a pH range of 0.22. The proteolytic component gave a further smaller fraction at pI 4.68, representing 4 % of the total activity in a pH range of 0.23.

2.4. Ampholine

Since in the focusing of these enzyme preparations the absorption curves of the fractions at 280 nm showed a smaller maximum in the most highly alkaline region and a very high peak in the most acid region at which no enzyme activity was detectable, a control run was performed with 1 % of Ampholine between pH 3 and 6. The O.D. at 280 nm and conductivity of the fractions were measured. The results are shown in Fig. 9.

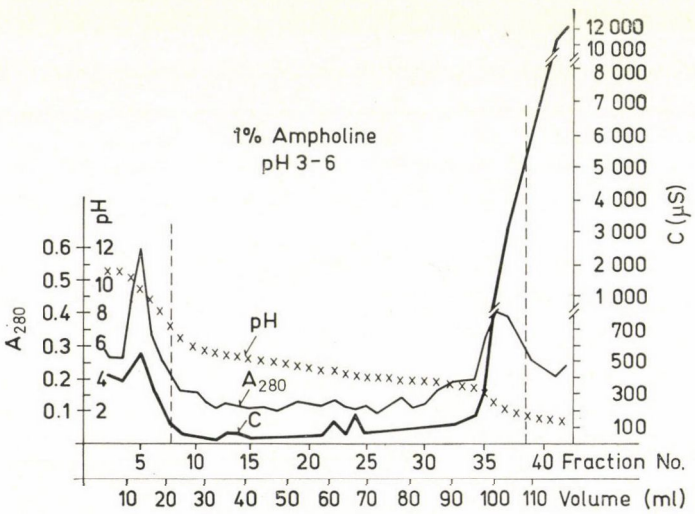

Fig. 9. Fractionation of Ampholine, pH 3 to 6, without protein. The concentration of Ampholine: 1%. A_{280} = absorption measured at 280 nm; C = conductivity (μS

It can be seen in the figure that there is a high absorption peak between pH 6 and 10.5, as well as between pH 1.4 and 3.4 associated, mainly in the strongly acid region, with high conductivity. The O.D. of Ampholine is low where the pH gradient is linear (between 3.4 and 6 pH) with almost identical conductivities of the fractions, thus Ampholine does not interfere with separation in the important pH range of the experiment.

3. Conclusions

Assessment of the results prove the applicability of the method to the determination of the isoelectric points of the milk-clotting and proteolytic components and of the location of the lipase component of the investigated enzyme preparations. When the pH gradient is appropriately narrowed, the main part of the enzymes gives a sharp peak within a pH range of 0.07 – 0.22, indicating the separability of protein components, the isoelectric points of which differ by not more than a few hundredths. This is particularly conspicuous when the preparation *E31* is run twice, for then in 3/4 of the total volume of the column the linear pH gradient lies between pH 3.25 and 4.25.

The method is well reproducible and the damage caused to the preparation is fairly slight, as from the majority of the preparations 50 to 80% of the applied enzyme quantity can be recovered (Fig. 4).

It occurred more than once in the course of our experiments that, after focusing, 105 to 190% of the applied enzyme activity were recovered (Table 4). This finding might be explained by the separation of some enzyme-activity-blocking agent(s) from the enzyme in the course of the focusing operation. VESTERBERG (1971b) made a similar observation in his study of enzymes obtained by the fermentation of *Staphylococcus aureus*.

Comparison of the investigated *Mucor* enzymes shows a great similarity between the isoelectric points of the main milk-clotting components of the two enzyme preparations produced by surface fermentation *(E31* and *MR)*. There is, however, a marked difference between *E31* and the preparation produced by submerged fermentation *(E32)*, not only with regard to their pI values, but also inasmuch as in the case of *E32* proteolytic enzyme activity is not associated with an absorption maximum at 280 nm which would characterize tyrosine and tryptophane, but has a smaller maximum at 220 nm. This suggests another composition of *E32* than that of *E31* and *MR*. These experiments support our earlier assumption, namely that, under the conditions of submerged fermentation, the mould *Mucor pusillus* Lindt synthetizes an enzyme of other composition and properties than the one synthetized in surface fermentation (POZSÁR-HAJNAL *et al.*, 1974 a,b).

In addition, under the given conditions, the preparation *E32* appeared to be highly sensitive. We could only recover 18 to 24% of the proteolytic activity after focusing, while milk-clotting and lipase activities were deteriorated to a degree that they could not be detected or were detected in very small quantities only. This enzyme behaved differently from the others also when fractionated on an ion exchanger or by some electrophoretic technique (MORVAI, 1971; VÁMOS-VIGYÁZÓ & MORVAI-RÁCZ, 1970; VÁMOS-VIGYÁZÓ *etal.*, 1973).

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The authors wish to express their thanks to Dr. I. KISS, head of department and to Mrs. G. KOVÁCS-PROSZT, senior research worker, for their useful advice and assistance.

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Acta Alimentaria, Vol. 4. (1), pp. 81—94 (1975)

MORPHOLOGICAL CHARACTERISTICS OF THE SPORE HEAD OF *PENICILLIUM PURPUROGENUM* AS AFFECTED BY GAMMA IRRADIATION

E. NYERGES-ROGRÜN

(Received December 22, 1973)

Upon irradiation in the moist state with 150 krad, tne conidia of *Penicillium purpurogenum* formed a progeny with brushes identical to those of the control. When irradiated with 250 krad, the metulae of the brushes were elongated and upon treatment with 350 krad the brushes were rudimentary.

Morphological changes were observed also at 150 krad when irradiation was combined with heat treatment. The morphological changes upon various combined treatments became apparent in the elongation of the metula and of the sterigma, in the reduction of their numbers, in the formation of the so-called sub-head, in the irregular separation of the conidia from the metula and in other deformations.

After fractionated irradiation the brushes are deformed to such an extent that their elements are hardly recognizable.

In preliminary experiments in collaboration with FARKAS and co-workers (1972), the microbial flora of bread, strawberry, tomato and grapes irradiated with various doses, was investigated. Of the fungi selected after radiation treatment, the progeny of the *Botrytis* species grown on nutrient, were found to be deformed. These observations induced us to investigate the morphological changes (POMPER & ATWOOD, 1955) occurring in fungi upon irradiation with increasing doses. In the study of complex radiation effects, the establishment of lethal doses may be affected not only by the change in sporulation (WEBB *et al.*, 1969; BUCKLEY *et al.*, 1969; SOMMER *et al.*, 1967, 1972) and by the reduction of toxin production (WILSON & WILSON, 1964), but also by the modification of the morphological characteristics.

1. Materials and methods

1.1. Test fungi

The preliminary experiments were performed on 3 fungal species *(Aspergillus, Penicillium, Botrytis)* of the mould collection of the *Research Institute for Viticulture and Enology*, originally selected from grapes. The conidia of these species were irradiated with various doses and the changes appearing in the progeny were observed. *Penicillium purpurogenum* Stoll, strain No. 787, appeared to be a particularly suitable test material, therefore, in the detailed study, only this strain was used.

On the basis of the work of RAPER and co-workers (1949), the test fungus was found to belong to the *Biverticillata* group and its morphological characteristics showed it to be identical with *Penicillium purpurogenum* Stoll, grown on *Czapek* nutrient agar.

1.2. Radiation treatment

Irradiation was carried out in the 20 kCi RH-gamma-30 radiation source of the *Central Food Research Institute*, Budapest, at a dose rate of 870 krad h^{-1}. The doses applied were 100, 150, 200, 250, 300, 350 and 400 krad, respectively.

1.3. Treatment of the dry and moist conidia

The conidia of *Penicillium purpurogenum* grown on *Czapek* agar slants in test tubes were irradiated with 100, 200 and 400 krad, respectively.

Treatment A. The conidium mass of one test tube was suspended in 0.1 ml Tween-80 (0.02%) in 4 ml liquid and the moist conidia thus obtained were irradiated.

After 24 hours the moist conidia were sparged in *Czapek* agar, solidifying in a petri dish of 10 cm ∅. The replicates were prepared by plating the contents of a test tube in doses of 0.5 ml.

Treatment B. The colonies were taken out from the agar slant, and irradiated directly, then placed into a dry test tube. After 24 hours, the dry conidia of the colonies were streaked on agar, using Tween-80.

1.4. Microscopic study of the progeny

Of the colonies grown on *Czapek* agar, 30 colonies of each variant were selected. Aqueous preparation was prepared of every colony. Data related to 30—50 brushes per variant were summarized and the average established. If the change in size was observed in more than 50% of the brushes measured, this was considered a size alteration. Other deformations of the brushes were considered individually.

1.5. Combined treatment

Heat treatment was applied prior to irradiation.

Temperature, °C	Period of treatment, minute	Dose level, krad		
30	5	150	250	350
30	10	150	250	350
50	10	150	300	—

The conidia were prepared according to *Treatment A*. The progeny was investigated according to para. 1.4. in the course of 14 days.

1.6. Fractionated irradiation

The dry colonies *(Treatment B)* of the test strain were irradiated with 100 and 200 krad, resp., propagated, and the 10-day-old sporulating progenies were again irradiated with 100 and 200 krad, respectively. Thus the total dose applied was 200, 300 and 400 krad, respectively.

The progenies were studied as described in *Treatment B* and para. 1.4. for a period of 14 days.

2. Results

2.1. Effect of irradiation in Treatments A and B

Morphological differences were not observed between the progenies of *Treatments A* and *B* at doses of 100 krad.

Among the progenies of fungi treated with 200 krad, individual spore heads with elongated brush were found. Of the elements of the brush, the elongation of the metula was found characteristic. Instead of the size 10—14 μm $\times$ 2.5—3 μm, as described in the identification key, the metula was found to be longer by about 6—8 μm in the average, having a size of 18—20 μm $\times$ 3—3.5 μm. The number of metulae decreased from 5—7 to the average of 3—5 *(Treatments A* and *B)*. In some of the metulae an increased diameter and irregular shape were also observed *(Treatment A)*. The sterigmata, originally parallel, became divergent and their size increased by about 2—4 μm (Fig. 1/ *a* to *d*). After treatment with 400 krad, not a single conidium germinated.

2.2. Morphological alterations upon combined treatment

Heat treatment in itself caused no morphological changes in *Penicillium purpurogenum*; however, the combined treatment increased radiation sensitivity in the spores. The study of conidia given a heat treatment of 10 minutes at 30°C has shown a substantial increase in the number of germinated conidia in comparison with those treated only with 350 krad.

In the progeny of fungi given a heat treatment of 5 minutes at 30°C and a radiation dose of 150 krad, the metulae and sterigmata were found to be elongated. The average size of the metulae was 20—22 μm $\times$ 4—5 μm, while the size modification of the sterigmata was not consistent (Fig. 2/*a*). After treatment with 250 krad the elongation of the metulae increased, the number

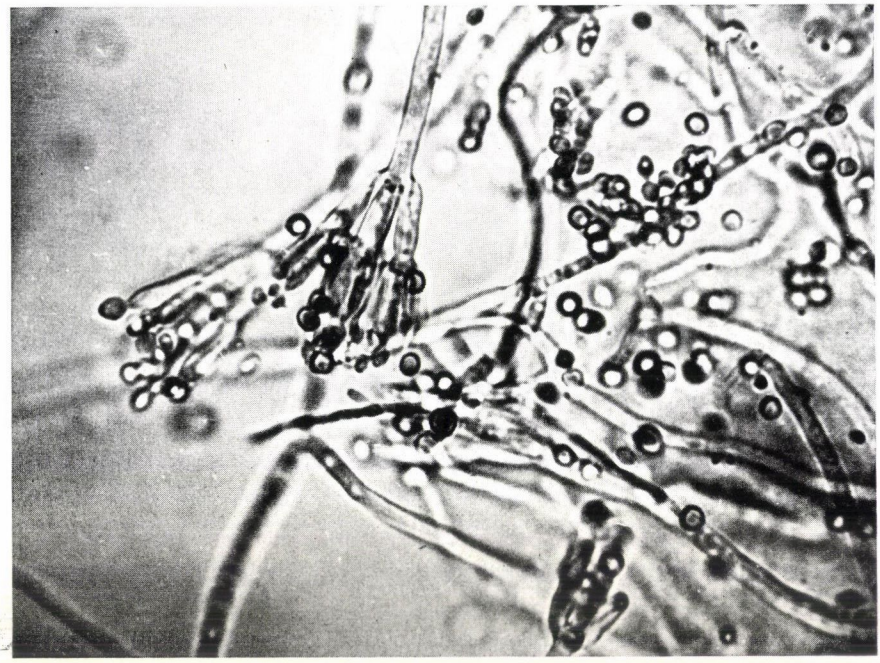

Fig. 1/b

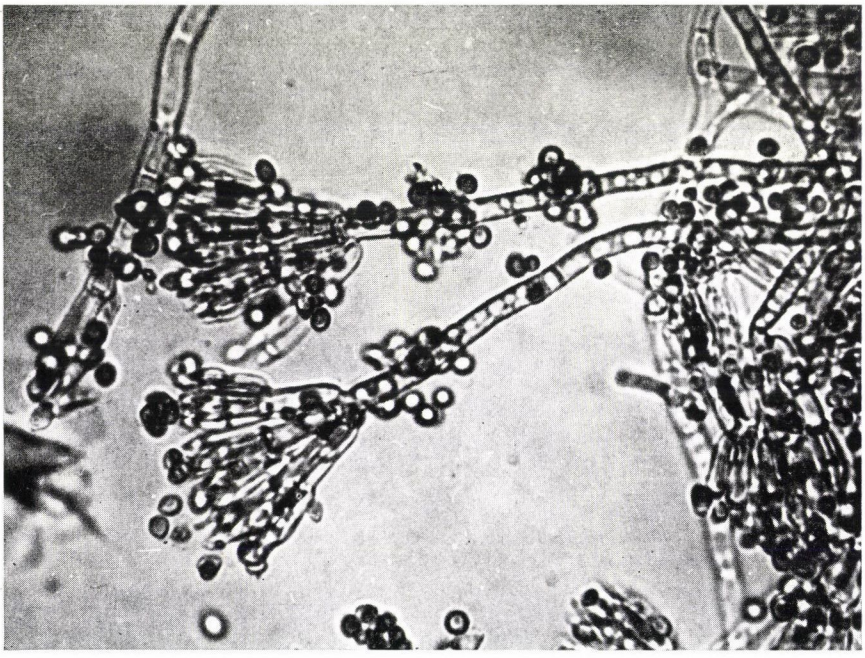

Fig. 1/a

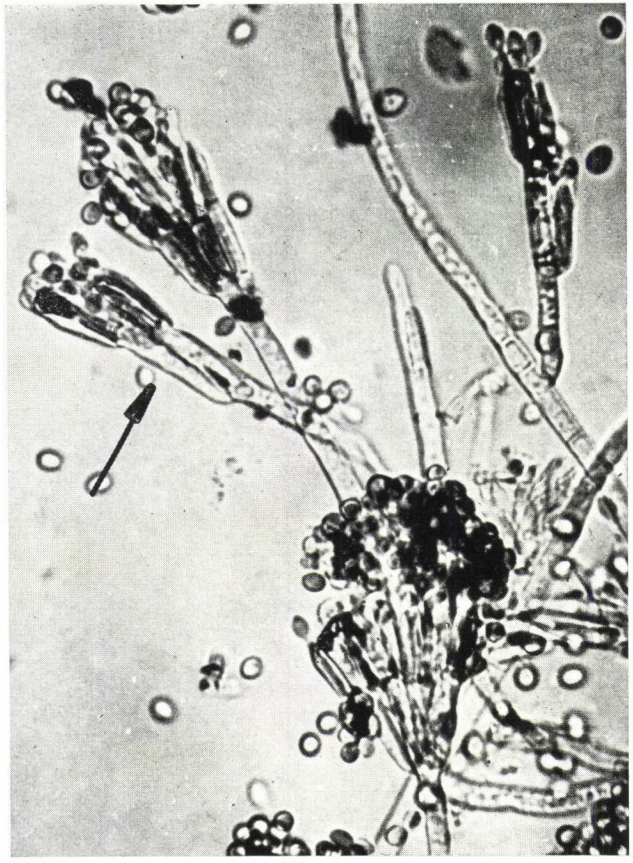

Fig. 1/c

Fig. 1/d

Fig. 1. Development of wet and dry conidia of *Penicillium purpurogenum* as affected by gamma radiation. *a* — Culture not irradiated (control); *b* — culture outgrown of conidia irradiated with 100 krad; *c* — culture outgrown of dry conidia irradiated with 200 krad; *d* — culture outgrown of wet conidia treated with 200 krad (irregular brush)

Fig. 2/b

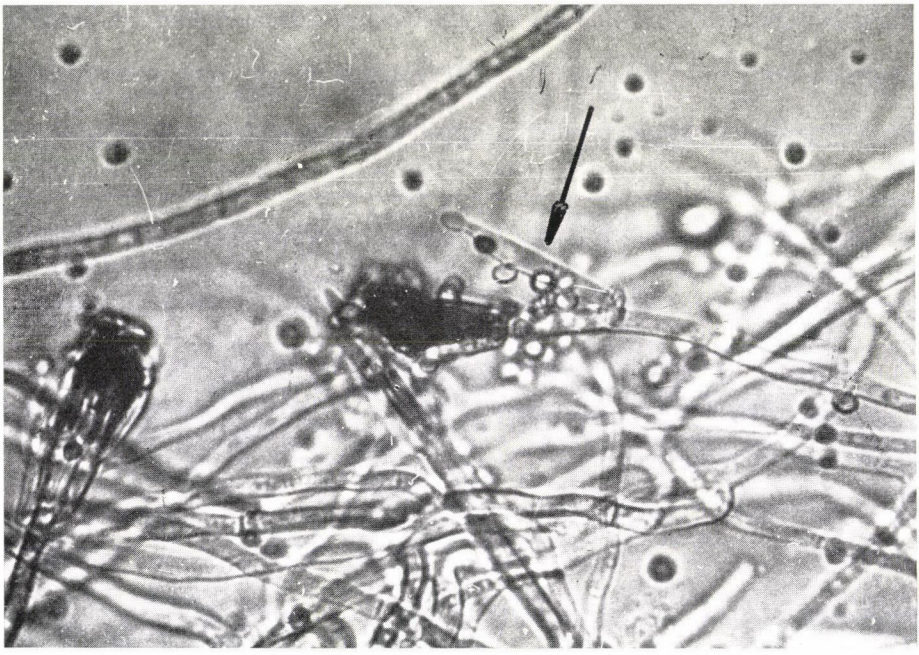

Fig. 2/a

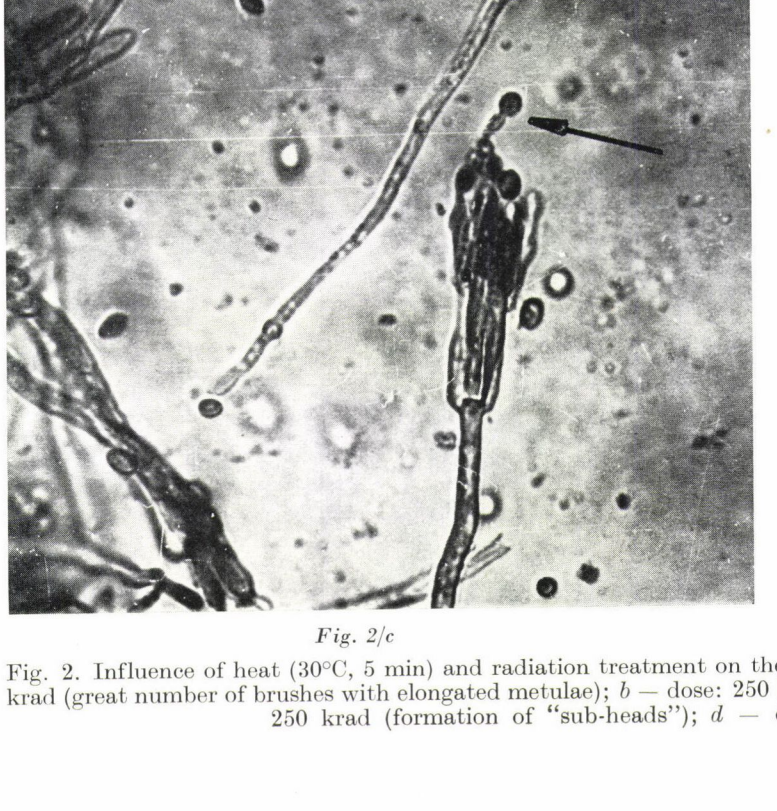

Fig. 2/c

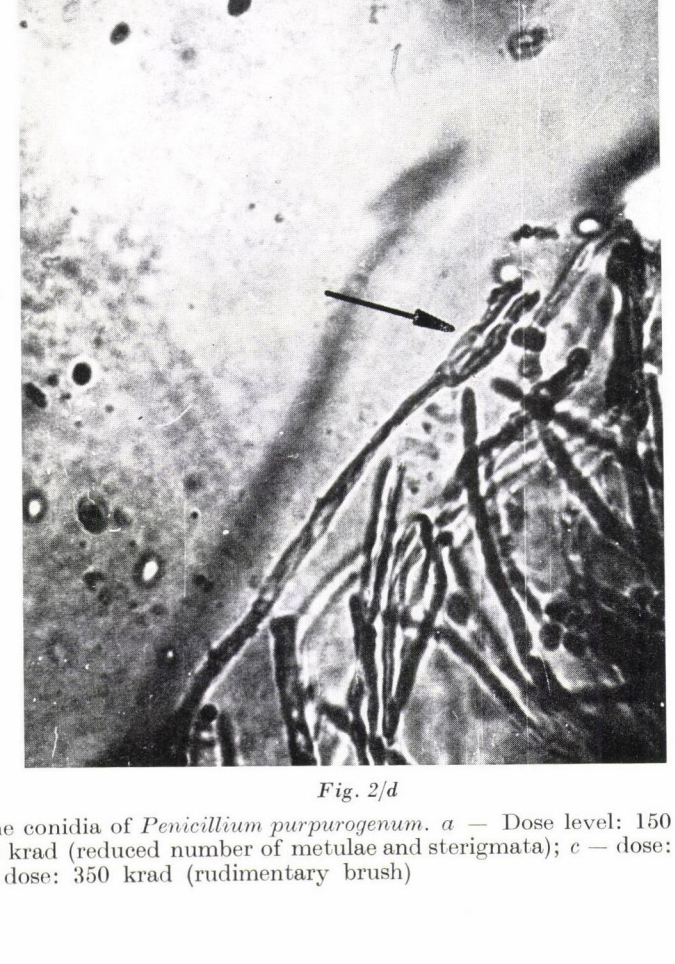

Fig. 2/d

Fig. 2. Influence of heat (30°C, 5 min) and radiation treatment on the conidia of *Penicillium purpurogenum*. *a* — Dose level: 150 krad (great number of brushes with elongated metulae); *b* — dose: 250 krad (reduced number of metulae and sterigmata); *c* — dose: 250 krad (formation of "sub-heads"); *d* — dose: 350 krad (rudimentary brush)

Fig. 3/b

(For legend see p. 90)

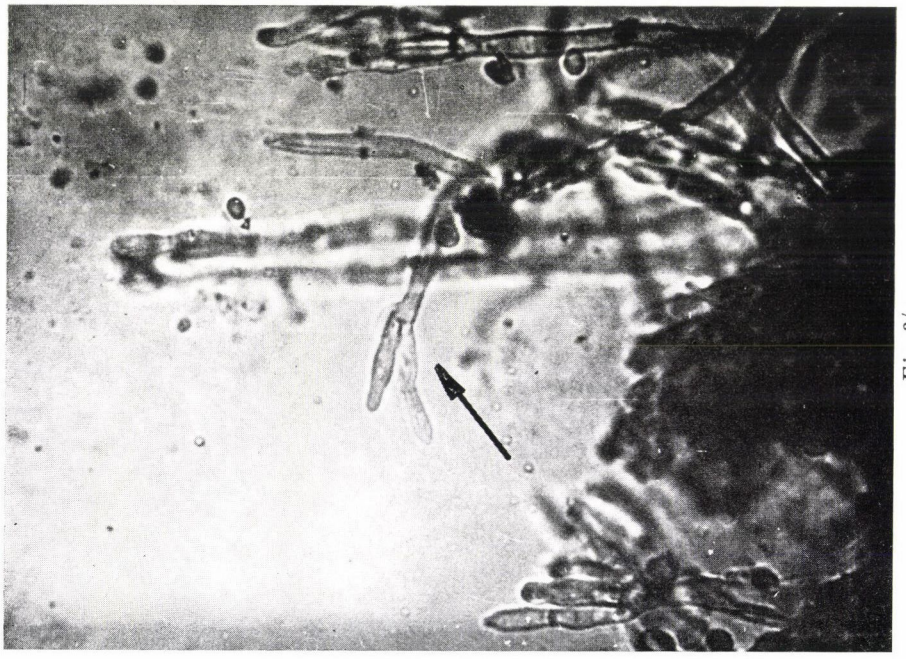

Fig. 3/a

Fig. 3/d

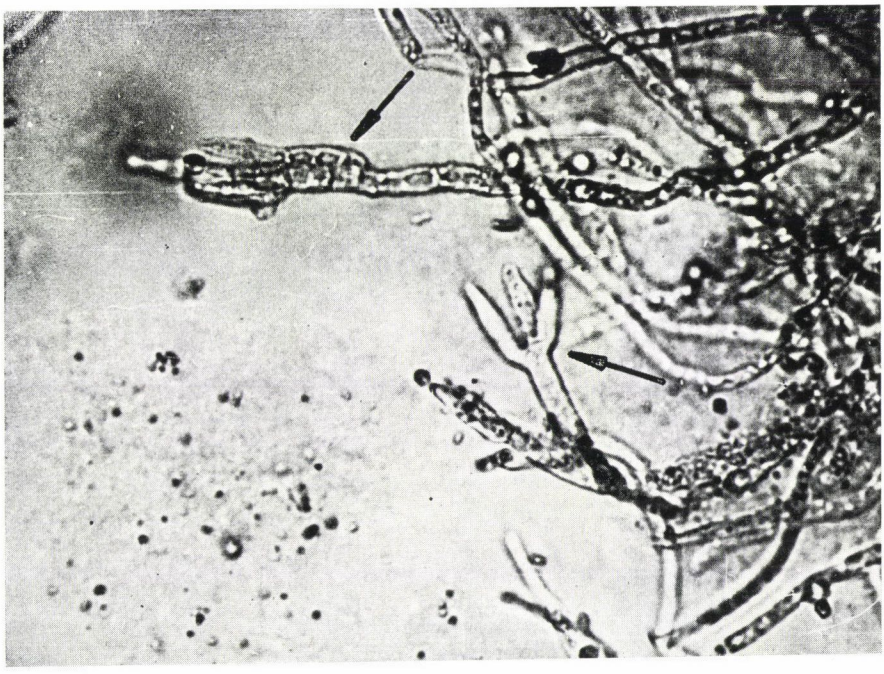

Fig. 3/c

(For legend see p. 90)

Fig. 3/e

Fig. 3. (For Figs. 3/a—3/d see pp. 88—89) Influence of heat and radiation treatment on the conidia of *Penicillium purpurogenum*. *a* — Heat treatment: 10 min at 30 °C and 150 krad (rudimentary brush); *b* — 10 min at 30°C and 250 krad ((conidium separating from metula); *c* — 10 min at 50°C and 150 krad (defective and deformed brush); *d* & *e* — 10 min at 50°C and 300 krad (elements of brush elongated, conidium not formed)

of metulae and sterigmata decreased (Fig. 2/*b*) and the formation of "sub-heads" was observed. (Fig. 2/*c*) Upon treatment with 350 krad, only a single rudimentary colony or brush was formed (Fig. 2/*d*).

After treatment at 30 °C for 10 minutes and with 150 krad the brush formed contained only a few elongated metulae and a single sterigma (Fig. 3/*a*). Following treatment with 250 krad, only rudimentary brush was formed. In some of the samples the conidium separated irregularly directly form the metula (Fig. 3/*b*). After treatment with 350 krad brushes were not formed at all.

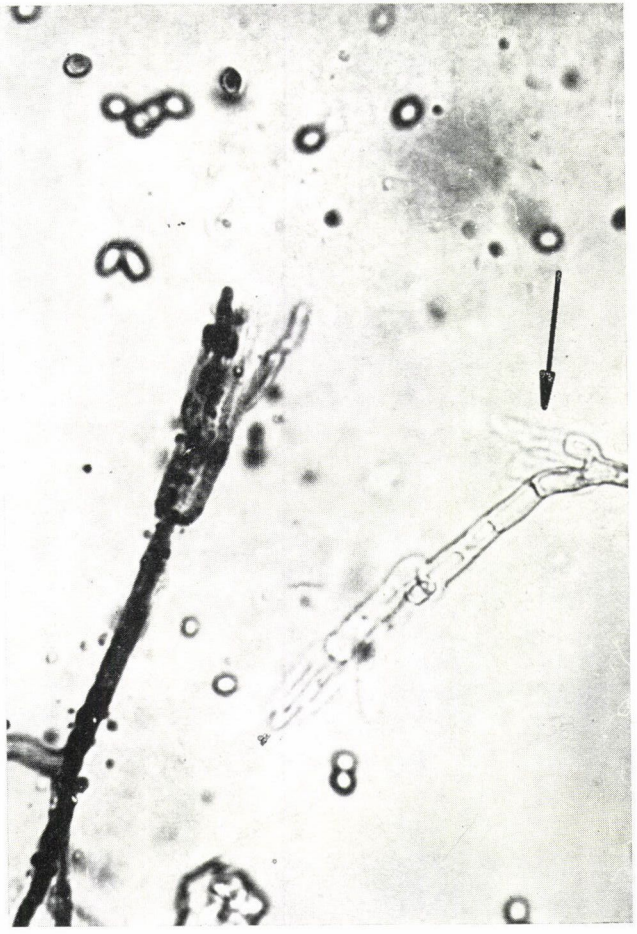

Fig. 4/a

Fig. 4/b

Fig. 4. Effect of fractionated radiation treatment upon the conidia of *Penicillium purpurogenum*. *a* — deformed brush developed upon treatment with a total dose of 100 + 100 krad; *b* — effect of a total dose of 100 + 200 krad

The spore heads of fungi given 10-minute treatment at 50°C and irradiated with 150 krad (Fig. 3/c) were characterized by deformed brushes and minimal conidium development. Upon treatment with 300 krad extremely elongated, completely deformed, defective brushes were produced (Fig. 3/*d* & *e*).

2.3. Effect of fractionated radiation treatment

Upon fractionated treatment with a total of 200 krad, the progeny developed elongated, yet recognizable brushes. Upon treatment with 300 krad, the brushes were several times the length of those of the control samples and completely deformed (Fig. 4/*a* & *b*). The dose of 400 krad had a lethal effect.

3. Conclusions

In these preliminary experiments it was not our aim to statistically evaluate the correlation between the increase of radiation dose and the germination (survival) of conidia, as shown earlier by WILSON and WILSON (1964), KOPELMAN and co-workers (1967) and observed by us, too.

The composition of the nutrient on which the conidia are streaked after irradiation is also of interest. We used *Czapek*'s medium because RAPER and co-workers (1949) identified *Penicillia* on this medium and the size data published by them served as a basis for comparison. BUCKLEY and co-workers (1969) and SOMMER and co-workers (1972) continued their investigations 24—48 hours after irradiation. During this period the cells regenerate to some extent. In our experience 24 hours are sufficient for this.

According to WEBB and co-workers (1969) and SOMMER and co-workers (1972), the moisture content of spores or conidia prior to irradiation, as well as in the developmental stage of the mycelia, affects the radiation resistance of fungi. In our experience conidia irradiated in the wet state proved to be more radiation-sensitive than those treated in the dry state and the brush developed by the former differed substantially from that of the control.

Radiation treatment combined with heat treatment (KISS & CLARKE, 1969; ROY *et al.*, 1972; SOMMER *et al.*, 1972) is more effective than radiation treatment in itself. In our experience the combination of these two treatments has far-reaching morphological and biological effects.

It is of interest to mention that the metabolism of the fungus was also affected by the combined treatment. *Penicillium purpurogenum* is known to produce a deep-red pigment, *purpurogenon* diffusing into the nutrient (TURNER, 1971). This pigment was produced by the test species after 5-minute treatment at 30°C and irradiation with 150 krad, in an amount equal or nearly

equal to that produced by the control (measured with *Unicam* spectrophotometer at 420 and 520 nm). After treatment with 250 krad, however, the solution was only pale pink.

Radiation sensitivity or the lethal radiation dose is characteristic of the species (MOHYUDDIN & SKOROPAD, 1969; WEBB *et al.*, 1969). In our study, too, *Botrytis*, *Aspergillus* and *Alternaria* species and certain yeasts were more resistant to radiation treatment than *Penicillium purpurogenum*. A radiation dose of 400 krad proved to be lethal for the latter.

In conformity with the findings of POMPER and ATWOOD (1955), it was established that the morphological changes increased proportionally with increasing radiation doses. In order to confirm the observations, we varied the radiation doses and applied combined treatment.

Fractionated treatment caused similar, in certain cases more excessive morphological changes than the same dose in a single treatment.

*

The author wishes to thank Dr. I. KISS for performing irradiation and his valuable advice and Mrs. A. ROSZMUSZ for her devoted technical assistance.

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Acta Alimentaria, Vol. 4. (1), pp. 95—112 (1975)

ATTEMPTS TO INCREASE STORAGE STABILITY OF STRAWBERRY YOGHURT BY COMBINATION TREATMENTS

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(Received July 18, 1974)

The aim of the experiments was to establish whether the microbiological stability of strawberry yoghurt might be improved by decreasing the microbial load of the fruit.

The effect of heat treatment, freezing, irradiation and various combinations of these treatments upon cell count and sensory quality was investigated. It was established that none of the individual treatments was entirely satisfactory. Surfacial heat treatment at 55 °C, freezing and irradiation with 0.4—0.6 Mrad substantially increased the storage life of strawberries or that of the yoghurt prepared with this fruit; when compared to yoghurt made with frozen strawberries by the dairy factory, the increase was 2.5fold at 15 °C and 3.5fold at 2 °C. The relative increase of storage life was lower at lower yeast-cell counts.

The strawberries irradiated with doses above 0.2 Mrad showed aroma and flavour changes immediately upon treatment. This effect, however, was eliminated after some days. The yoghurt made with strawberries given a radiation treatment of 0.57 Mrad did not differ organoleptically from the yoghurt made with untreated strawberries.

In the knowledge of the survival rate of yeasts after irradiation the D_{10} values were established. These were found in the dose range between 0.043 and 0.087 Mrad.

It was established that the applied heat treatment, freezing and irradiation at these dose levels and at $10^3 - 10^4$ cells per gram were not sufficient from the point of view of microbiological stability.

In accordance with up-to-date trends in nutrition it is desirable to reduce the proportion of foods rich in calories while increasing the proportion of foods rich in protein. Milk and dairy products play an important role in achieving this aim. This is the reason for the increasing popularity in Holland of yoghurt containing fruit.

The storage life of this product at refrigerator temperatures (3 to 5 °C) is relatively low. However, the symptoms of spoilage appear sometimes even before the end of this period. Spoilage is caused in most cases by yeasts. The yeasts are introduced either with the fruit or the contamination occurs in the course of production. Along with the fruit, beet sugar is added to the yoghurt. The proportion of the latter is 8%. If the temperature of the product rises above the given level, the activity of the yeasts becomes enhanced and this has to be taken into account. Since the fruit is stored frozen and receives heat treatment prior to utilization, only the yeasts surviving freezing and heat treatment present a problem.

The aim of these experiments was to study the possibility of reducing the viable yeast count in strawberries by radiation treatment, by investigating combination treatments (heat treatment, freezing) permitting the extension of the storage life of strawberry yoghurt, while maintaining the quality of strawberries.

1. Materials and methods

1.1. Preservation of strawberries

1.1.1. Materials. The *Senga-Sengana* strawberry variety was used in the experiments. Stem and sepal were removed in the processing plant and the fruit was kept in storage at 10 °C.

1.1.2. Heat penetration measurements in strawberry. Strawberries are highly sensitive to heat treatment, therefore the changes in the quality of strawberries caused by reaching the required core temperature were investigated. Measurements were carried out with a thermistor of 2 mm diameter at 55, 65 and 75 °C, respectively. The calibration curve of the thermistor was determined in an ultrathermostat (*Grant Instruments*, Cambridge, Type SB 4), filled with demineralized water, for the measurement of the change in resistance a measuring bridge (*Philips*, GM 4144) was used in the temperature range of 27.5 to 83.0 °C. The logarithm of the resistance was plotted as a function of temperature and the equation of the regression curve was established. On the basis of this calibration curve, the temperature in the cortex of strawberries as a function of the time of heat treatment was plotted, taking into account the size of the fruit and its weight.

1.1.3. Preservation experiments. Two experimental series were made. The effect of washing, heat treatment and freezing in combination with various radiation doses was investigated. At the same time, the effect of radiation treatment alone was also studied, though no substantial increase of storage life could be expected. In order to be able to observe the effect of the various treatments, an attempt was made to increase the initial viable cell count of the strawberries. After washing, contamination and heat treatment the strawberries were packed in polyethylene pouches, 200 g in each.

In the first experimental series the effect of irradiation, washing, heat treatment and freezing was studied individually and in combination. Contaminated strawberries were exposed to the same treatments. Heat treatment was provided by immersing the fruit in a water bath of 55 °C for 2 minutes. For the freezing experiments the fruit was packed in pouches and frozen at —30 °C. Irradiation was carried out after this.

In the second experimental series a 3-minute heat treatment was given at 50 °C and 1 minute at 55 °C. In this series only the contaminated strawberry samples were exposed to the different treatments or their combination.

1.1.4. Contamination of strawberries with yeasts. To increase the yeast cell count in the strawberry, the samples were immersed for two minutes in a yeast suspension. The cell count of the suspension was $\sim 10^6$ ml^{-1}, thus the initial yeast cell count of the sample could be increased by one order of magnitude. The contaminating microorganism was isolated by *Dr. H. Labots* (*Nederlandse Melk Unie N.V., Central Laboratorium*, Breukelen) from a Bulgarian type yoghurt made with strawberries. The yeasts were propagated in liquid shake cultures in Erlenmeyer flasks at 25 °C. [Composition of the nutrient: 5.0% glucose (*Merck*); 0.1% pepton (*Oxoid*); 0.1% yeast extract (*Oxoid*); pH 3.6—3.8 (adjusted with lactic acid); sterilization: 15 minutes at 115 °C. Shaking apparatus: *Marius*, Utrecht, Type 66 SS, number of strokes 200 min^{-1}, length of stroke 10 mm.] After a growth period of 20 hours the total cell count was $\sim 10^8$ ml^{-1}. The culture was suspended in tap water thereby attaining a final concentration of 10^6 cells per ml.

1.1.5. Irradiation. Irradiation was carried out with the ^{60}Co radiation source of 200 000 Ci nominal activity of the Pilot Plant for Food Irradiation, Wageningen. In the first experimental series 0.18, 0.38 and 0.57 Mrad doses were applied, in the second 0.18 and 0.36 Mrad doses were applied at a dose rate of 0.2 Mrad h^{-1} in both series. The doses absorbed were established with polymethylmethacrylate (PMMA-perspex) dosimeter or with the aid of a spectrophotometer (CHADWICK, 1970).

During irradiation the temperature of the unfrozen samples was between 25 and 28 °C and that of the frozen samples at —30 °C. The latter samples were kept in polystyrene cases cooled by cooling flasks.

1.1.6. Storage of the samples. After treatment, the samples not frozen were stored at 2 °C, while the frozen samples were kept at —23 to —25°C.

1.2. Determination of the viable yeast count

The samples to be processed were stored at 2 °C for about 16 to 18 hours, then weighed with 0.1 g accuracy. They were diluted to 200 ml in a solution of 0.85% NaCl and 0.1% pepton (*Oxoid*) and homogenized in an MSE *Atomix*, first for 30 sec at 6000 rpm, then for 50 sec at 12 000 rpm. Immediately upon treatment or in the initial stage of storage, 10-fold dilutions were made of the basic suspension and the cell count was established by using pour-plate technique and potato–dextrose–agar (*Oxoid*) nutrient. In later stages of storage, the Most Probable Number (MPN) technique was used to determine the cell counts. (5×10 ml, 5×1 ml, and 5×0.1 ml of the basic suspension was inoculated in the nutrient as described under para. 1.1.4. For 10 ml of inoculum a nutrient of double strength was used.) Propagation was carried out at 25 °C for 6 days. Applying the MPN technique, the positive samples were controlled under the microscope.

1.3. Sensory tests

The organoleptic tests were carried out by a panel of 5 to 7 members, ranking for significance of difference the multiple comparators (KRAMER, 1956). The samples were ranked and the significance of differences between rank sums was calculated by using KRAMER's tables (KRAMER, 1960). When the taste of strawberry was tested, the ranking was carried out after scoring. A 10-value scoring system was applied, where:

9—10 scores: excellent,
7— 8 scores: good,
5— 6 scores: medium,
4 scores: acceptable,
3 scores: objectionable,
1— 2 scores: bad.

We established a difference in flavour between strawberry yoghurt prepared with irradiated, and strawberry yoghurt prepared with unirradiated strawberries. These samples were compared by the triangular test method (BENGTSSON, 1953).

1.4. Preparation of strawberry yoghurt and investigation of its storage life

The storage life of strawberry yoghurt prepared with differently preserved strawberries was compared.

In the first experiment the reference sample was frozen in the dairy factory and was heat treated at 70 °C for 5 minutes before use. The other samples were heat treated at 55 °C for 1 minute, frozen and irradiated with 0, 0.18 and 0.36 Mrad. In the second experiment the following treatments were applied: (a) control: frozen by us, before use heated at 70 °C for 5 minutes; (b) frozen and irradiated with 0.57 Mrad; (c) heated at 55° C for 2 minutes; frozen and irradiated with 0.57 Mrad.

1.4.1. Preparation of strawberry yoghurt. The factory-made plain yoghurt was homogenized, then mixed according to the customary formula (1 000 ml yoghurt, 100 g sugar, 100 g strawberries). In accordance with the industrial practice, the strawberries were heat treated for 5 minutes at 70 °C prior to use, then homogenized in an MSE *Atomix* for 60 seconds at 12 000 rpm. The mixture thus obtained was then homogenized with an aliquot of yoghurt for 90 seconds, finally thoroughly mixed with the total amount of yoghurt. The strawberry yoghurt thus prepared was then filled into bottles of 125 ml capacity, previously sterilized (2 atm, 20 min) and closed with aluminium foil. The samples were stored at 2 and 15 °C, respectively. Each sample was made in 10—15 replicates.

1.4.2. Investigation of storage life. The beginning of fermentation is indicated by the appearance of gas bubbles. With increasing intensity of fermentation, the number and size of the bubbles increases. In the case of high activity, the gas formed bulges on the aluminium foil, loosens it and often the yoghurt or serum overflows. Since gas formation is easy to observe, this was considered the sign of spoilage and was plotted as a function of storage time. The time during which 20% of the samples underwent spoilage was considered the storability value. The relative keeping quality is the quotient of the times needed for 20% spoilage of the experimental and control samples.

1.4.3. Determination of the viable yeast count in strawberries. The same method was applied as under 1.2.

1.5. Determination of the radioresistance of yeasts isolated from strawberries

In order to be able to estimate the dose required to reduce the cell count of strawberries, the radiation sensitivity of several yeast strains isolated from strawberries was tested. Some of these strains originated from fresh strawberries:

Cryptococcus laurentii var. *flavescens* FRI-I-F230 (L2),
Metschnikowia reukafii FRI-I-S240 (L6),
Rhodotorula glutinis FRI-I-F368 (L7).

These strains were isolated by J. A. BARNETT (BUHAGIAR & BARNETT, 1971).

The author has also isolated strains from fresh strawberries. These, however, have not been identified yet and were marked *KS31* and *KS32*. Two strains isolated from strawberries treated with 0.57 Mrad were marked *KS33* and *KS34*, respectively.

Cultivation was carried out in the nutrient described under para. 1.1.4. at 15 and 20 °C (the first four strains were propagated at 15 °C). The yeast cells were separated from the nutrient by centrifuging for 30 minutes at 2 °C [*Christ (Osterode, Harz)* Type UJ 15, 3000 rpm]. The yeast cells were then suspended in the peptone—NaCl solution, centrifuged again and a suspension of 10^9 ml^{-1} cell count was prepared with peptone—NaCl solution (pH = 5.0).

1.5.1. Irradiation of the yeast cell suspension. Irradiation was carried out with the ^{60}Co source of 200 000 Ci nominal activity of the Pilot Plant for Food Irradiation. Radiation doses of 0.052, 0.105. 0.21 and 0.42 Mrad were applied at a dose rate of 1.82 Mrad h^{-1}. The absorbed doses were determined with PMMA-perspex.

1.5.2. Determination of the surviving cells. After radiation treatment tenfold dilutions were made and the surviving cell count was established of the

first four strains, as described under para. 1.5., by surface-plate technique and in every other case by pour-plate technique, on potato—dextrose—agar nutrient medium. 3 to 9 samples were counted at each dose level. The average count of colonies and the standard deviation were established. The regression curve between the number of surviving colonies and the dose level was calculated and the D_{10} value was determined.

2. Results

2.1. Heat penetration measurements

On constructing the calibration curve of the thermistor, an extremely close correlation was found between temperature increase and the decrease of resistance ($r = -0.9994$, $n = 55$). On the basis of this, the core temperature of the strawberry was determined as a function of time, taking into consideration the size and the weight of the strawberries. The rate of temperature increase in the strawberries, starting from room temperature (22—23 °C), depends on the size and weight and is relatively even (Fig. 1).

As seen in the figure, when heated in a water bath, a relatively long period is needed for the strawberry to reach the required temperature. This takes 6—12 minutes, dependent on the size of the fruit. During this time the consistency, colour, taste and odour of the fruit changes completely, the colour and odour looses its character. Knowing this, we did not aim at increasing the core temperature of the fruit, we gave only a surface heat treatment.

2.2. Viable cell count of the strawberries

The viable cell count determinations showed the rinsing to reduce the cell count by one order of magnitude and the freezing at —30 °C gave a reduction of about 25% (Table 1).

Table 1

The viable cell count of strawberries as a function of treatment

Treatment	Viable cell count g^{-1}
unwashed	$(4.2 \pm 1.9) \cdot 10^4$
washed	$(4.4 \pm 2.0) \cdot 10^3$
frozen	$(1.1 \pm 0.6) \cdot 10^3$

Since the initial cell count was found to be low, we attempted to increase it by contamination. We succeeded in increasing it by nearly one order of magnitude. In the case of the contaminated strawberries a 2-minute treatment at 55 °C reduced the viable cell count by 86.7 % and freezing by more than 90 %. The combination of these two treatments resulted in a reduction of 3 orders of magnitude. Since treatment with the combination method and irradiation with 0.38 Mrad was very effective, the surviving fraction was determined with the MPN technique instead of the pour-plate technique. Treatment with 0.18 Mrad caused a reduction of one order of magnitude (Table 2).

Table 2

The viable cell count (g^{-1}) of strawberries as a function of treatment and irradiation dose

Dose (Mrad)	Treatment		
	Heat treated (55°C, 2 min)	Frozen	Heat treated (55°C, 2 min) + frozen
0	$(1.3 \pm 0.2) \cdot 10^3$	$(6.9 \pm 6.1) \cdot 10^2$	3.6 ± 1.7
0.18	$(1.7 \pm 0.6) \cdot 10^2$	$(9.7 \pm 0.8) \cdot 10$	< 1
0.38	< 1	4.6 ± 2.3	< 1
0.57	< 1	< 1	< 1

After two weeks of storage at —25 °C the samples contaminated with yeasts showed a further reduction of the viable cell count by nearly one order of magnitude. After 79 days the cell count of samples non-irradiated and irradiated with 0.18 Mrad was found to be below 2, when determined with the MPN technique. In the samples irradiated with 0.38 and 0.57 Mrad, respectively, the presence of viable cells could not be shown with sufficient reliability.

The strawberries used in the second experimental series had a higher initial cell count and were of poorer consistency than those of the first series. The initial cell count was increased by contamination to 10^4 g^{-1}.

The effect of freezing did not differ from that of the earlier experiments. The samples treated for 3 minutes at 50 °C and for 1 minute at 55 °C and then frozen, gave similar results. Compared to the samples that were only frozen, the reduction was about two orders of magnitude better. A radiation dose of 0.18 Mrad reduced the cell count of the frozen samples by more than one order of magnitude (Table 3).

Higher radiation doses combined with freezing gave such low cell counts as to be considered unreliable. After two weeks of storage the cell count was reduced by about one order of magnitude. After 72 days of storage at —25 °C, the cell count of samples treated for 1 minute at 55 °C and not irradiated was

Table 3

Changes in the viable cell count (g^{-1}) of frozen, and heat treated plus frozen strawberries, resp.

Dose (Mrad)	Frozen	Heat treated and then frozen	
		heat treated at 50°C: 3 min	heat treated at 55°C: 1 min
0	$(1.5 \pm 0.5) \cdot 10^4$	$(8.4 \pm 1.4) \cdot 10^2$	$(6.2 \pm 2.3) \cdot 10^2$
0.18	$(4.9 \pm 2.5) \cdot 10^2$	< 10	< 10
0.36	< 10	< 10	< 10

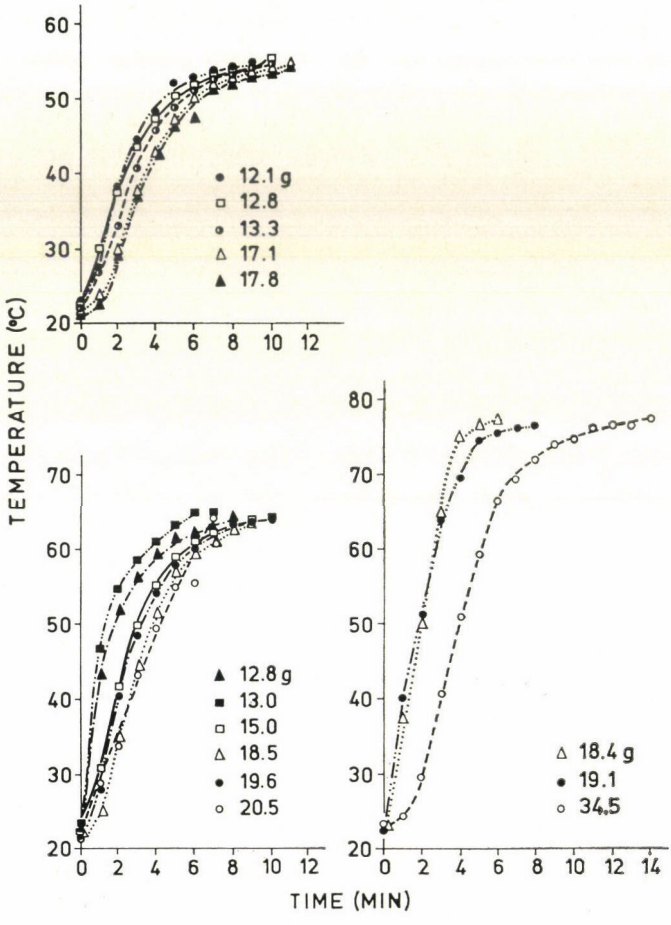

Fig. 1. The development of the temperature in the centre of the strawberry as a function of the heat treatment time (heated in water)

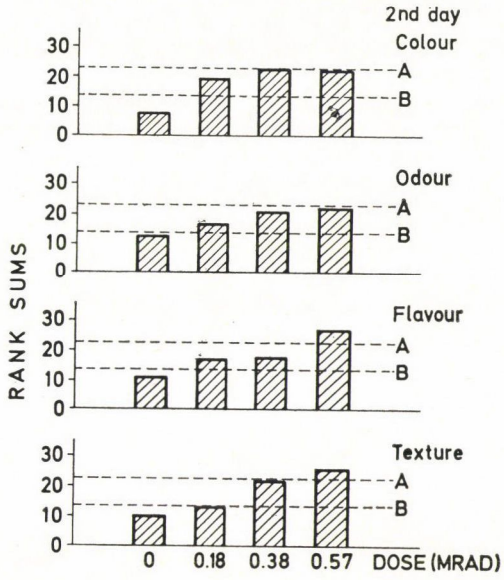

Fig. 2. Results of the sensory test on fresh strawberries as a function of radiation dose on the 2nd day of storage. The samples were stored at 2 °C. Rank sums between A and B do not differ significantly (95 per cent probability level. No. of panelists = 7)

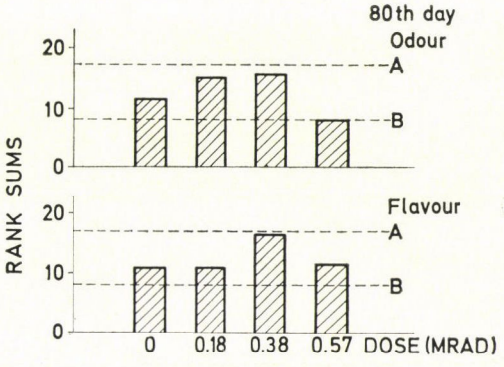

Fig. 3. Results of the sensory tests on heat treated (55 °C, 2 min) and frozen strawberries as a function of radiation dose at the 80th day of storage. The samples were stored at −25°C. Rank sums between A and B do not differ significantly (95 per cent probability level. No. of panelists = 5)

0.5—2.6 per gram, the count of samples irradiated with 0.18 Mrad was between 0.04 and 0.4 per gram. The initial 10^2 g^{-1} cell count of industrially frozen strawberries was reduced by a heat treatment of 5 minutes at 70 °C by nearly one order of magnitude.

2.3. Sensory evaluation of irradiated strawberries

In the first experimental series the sensory characteristics as a function of radiation dose of the strawberries irradiated in the fresh state were ranked, on the second day after treatment. The judges considered the untreated strawberries significantly better than those treated with 0.18, 0.38 and 0.57 Mrad, respectively (Fig. 2).

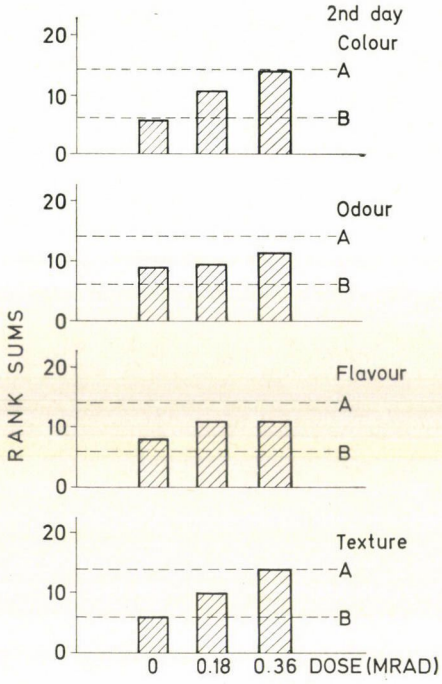

Fig. 4. Results of the sensory tests on fresh strawberries as a function of radiation dose on the 2nd day of storage. The samples were stored at 2° C. Rank sums between A and B do not differ significantly (95 per cent probability level. No. of panelists = 5)

After two weeks of storage the difference between samples frozen or samples treated for 2 minutes at 55 °C and frozen but not irradiated and those irradiated was not significant at the probability level of 95 %.

On the 80th day of storage the samples heat treated for 2 minutes at 55 °C and frozen in combination with irradiation, were judged to be similar in the sensory tests (Fig. 3).

In the second series of experiments treatment with 0.18 and 0.36 Mrad was applied. In the case of fresh strawberries the difference, in contrast to the earlier experiments, was not so pronounced, though with increasing doses a similar tendency became apparent (Fig. 4).

On the 5th day of storage the observations were similar to those made on the first experimental series, the difference between untreated and treated samples could not be proven. The tests carried out on the 72nd day confirmed these observations.

2.4. Storage life of strawberry yoghurt prepared with irradiated strawberries

Preliminary experiments showed that the various treatments or their combination with irradiation at 0.18 Mrad did not extend the storage life of yoghurt even at 2 °C.

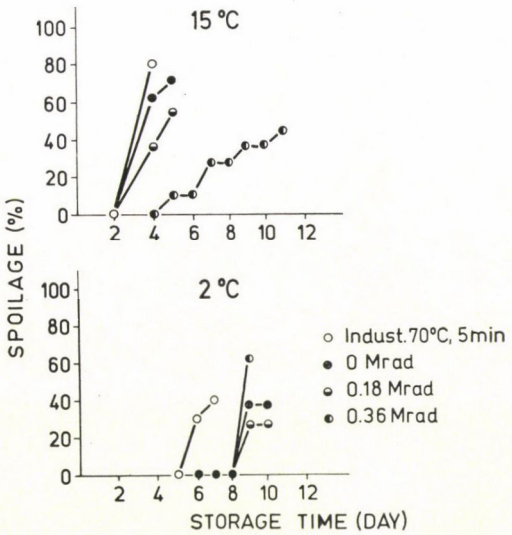

Fig. 5. Spoilage percentage of strawberry yoghurt as a function of treatment and storage time at 15 °C and 2 °C. The reference sample was frozen in the dairy factory and was heat treated at 70 °C for 5 min before use, the other samples were heat treated at 55 °C for 1 minute, frozen and irradiated

In the first experimental series strawberries industrially frozen and heat treated for 5 minutes at 70 °C prior to use, and strawberries heat treated as described under para. 1.1.3. at 55 °C for 1 minute and subsequently frozen and irradiated with 0.18 and 0.36 Mrad, respectively, were mixed with yoghurt. The samples thus obtained were stored at 2 and 15 °C. Of the samples stored at 15 °C those prepared with non-irradiated strawberries, were the first to show signs of spoilage. The next samples to spoil were those given radiation treatment of 0.18 Mrad; these were followed by the samples irradiated with 0.36 Mrad. Spoilage in this case must be ascribed to the microbial flora admixed with the strawberries, since the yoghurt and sugar had been homogen-

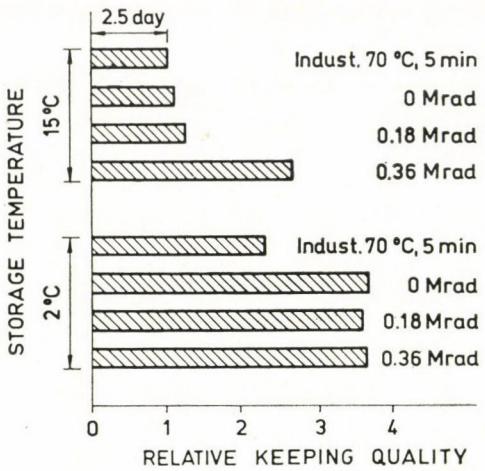

Fig. 6. Relative storage stability of strawberry yoghurt as a function of treatment and storage temperature. The reference sample was frozen in the dairy factory and was heat treated at 70 °C for 5 min before use, the other samples were heat treated at 55 °C for 1 minute, frozen and irradiated

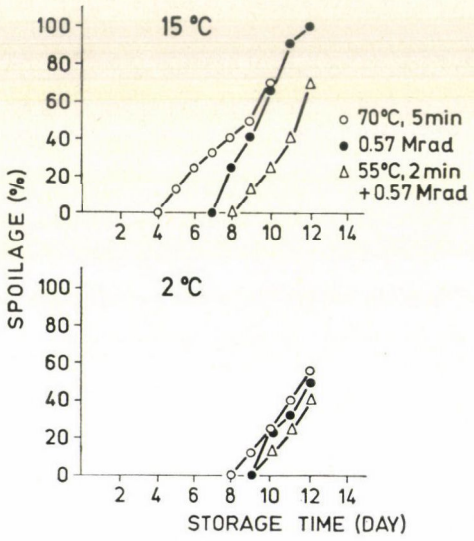

Fig. 7. Spoilage percentage of strawberry yoghurt as a function of treatment and storage at 15 °C and 2 °C. Following treatments were applied: (a) frozen (by us), before use heated at 70 °C for 5 min; (b) frozen and irradiated with 0.57 Mrad; (c) heated at 55 °C for 2 min, frozen and irradiated with 0.57 Mrad

ized and their contamination would have become apparent uniformly. In this case the spoilage showed a non-uniform distribution. The intensity of spoilage was observable foremost in the yoghurt samples stored at 15 °C. The gas formed during fermentation could be observed to bulge the aluminium caps. The sam-

ples stored at 2 °C showed less intense fermentation and less gas was formed (Fig. 5).

Evaluated on the basis of 20% spoilage of the samples stored at 15 °C those made with industrially frozen strawberries had a storage life of only 2.5 days, those treated with 0.36 Mrad could be kept for 6.6 days. Yoghurt receiving the same treatment, but stored at 2 °C had a storage life of 8.3 days. The data on relative keeping quality were summarized in Fig. 6.

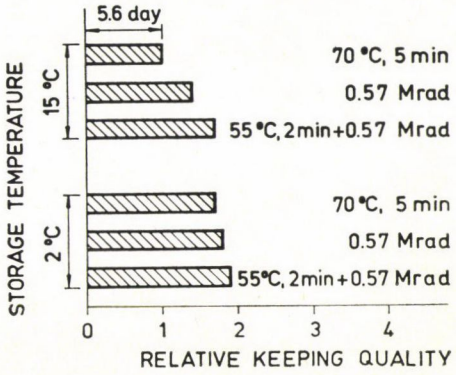

Fig. 8. Relative storage stability of strawberry yoghurt as a function of treatment and storage temperature. Following treatments were applied: (a) frozen by us, before use heated at 70 °C for 5 min; (b) frozen and irradiated with 0.57 Mrad; (c) heated at 55 °C for 2 min, frozen and irradiated with 0.57 Mrad

Since it was established in the first experimental series that strawberries treated with 0.36 Mrad may cause spoilage, in the second experiment the strawberries added to the yoghurt were (a) frozen and heat treated prior to use at 70 °C for 5 minutes; (b) frozen and given radiation treatment of 0.57 Mrad; (c) heat treated at 55 °C for 2 minutes, frozen and irradiated with 0.57 Mrad.

It was established that the microbiological stability of these samples was higher than in the first experiment. The storage life of the control was longer, too. The keeping quality of differently treated samples increased, but at most to double the life time at 2 °C, from 5.6 days to 10.7 days. The intensity of the spoilage at 0.57 Mrad was not quite as high, as at lower dose levels. The development of spoilage and the data on relative keeping quality are in Figs. 7 and 8. The viable cell counts of the strawberry can be estimated from our data to be about 0.01—0.2 per gram. The yeast was demonstrated from spoiled yoghurt under the microscope.

2.5. Sensory test of strawberry yoghurt

The sensory evaluation by triangular test of yoghurt made with strawberries frozen and heat treated for 5 minutes at 70 °C on one hand, and frozen and irradiated with 0.57 Mrad on the other, showed that the judges could

not differentiate (at the 95% probability level) between the samples. Out of nine judges the judgement of five was correct.

2.6. Radiation tolerance of yeasts isolated from strawberries

The survival of yeasts isolated from fresh and irradiated strawberries was investigated as a function of the radiation dose. From the data the regression curve was constructed and this was used to calculate the D_{10} values. These were found to be between 43 and 87 krad (Figs. 9, 10 and Table 4).

Table 4

D_{10} values of yeasts isolated from strawberries

Strain	D_{10} (krad)
Cryptococcus laurentii var. *flavescens* FRI-I-F230, (L2)	50
Metschnikowia reukafii FRI-I-S240, (L6)	45
Rhodotorula glutinis FRI-I-F368, (L7)	43
KS 31	43
KS 32	87
KS 33	53
KS 34	73

3. Conclusions

The initial cell count of fresh strawberries is 10^3—10^5 g^{-1}. The radiation dose suggested to extend storage life is 0.20—0.25 Mrad (JOHNSON *et al.*, 1965; FARKAS & KISS, 1967; LANGERAK, 1971; MAXIE *et al.*, 1971). By this treatment the useful storage life can be increased 2.5—3.0 times. However, a longer extension can not be achieved by this treatment, even if a storage temperature of about 0 °C is applied. It was shown in the present experiment that treatment with 0.57 Mrad is not sufficient either (Fig. 7). The storage life of yoghurt made with fruit is relatively low at refrigerator temperature (3—4 °C). In order to achieve microbiological stability of the product, the cell count of strawberries must be at the minimum.

According to these experiments, this aim can be achieved only by combined treatment. Contamination of strawberries is predominantly confined to their surface. Data on contamination of the cortex are to be found in the literature (ROMWALTER & KIRÁLY, 1939), however, these have not been confirmed. Knowing this, it is possible to reduce the cell count by mild surfacial heat treatment. It was found that heat treatment by immersion in water

caused an undesirable change of quality, if the core temperature was raised to the required level. If only surface treatment was given, no undesirable change occurred. A mild heat treatment combined with freezing resulted in the reduction of the cell count by about 3 orders of magnitude. A treatment with 0.36—0.57 Mrad ensured a further reduction of the cell count (Table 2).

In accordance with earlier observations (FARKAS *et al.*, 1972), the sensory tests show an undesirable change in the fresh strawberries immediately upon radiation treatment above 0.18 Mrad. However, in a few days' time this effect disappears even after a treatment with 0.57 Mrad. When tested by scoring, the samples exceeded the acceptability level and after ranking no significant difference could be found at the 95% probability level between untreated and irradiated samples (Figs. 2—4).

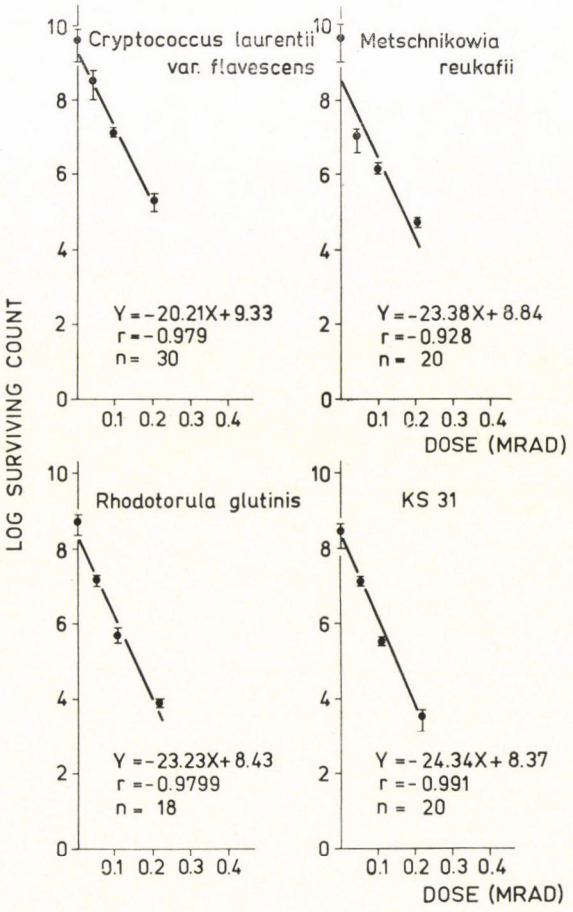

Fig. 9. The survival curves of yeasts as a function of radiation dose. (The vertical lines belonging to the mean values indicate the standard deviation)

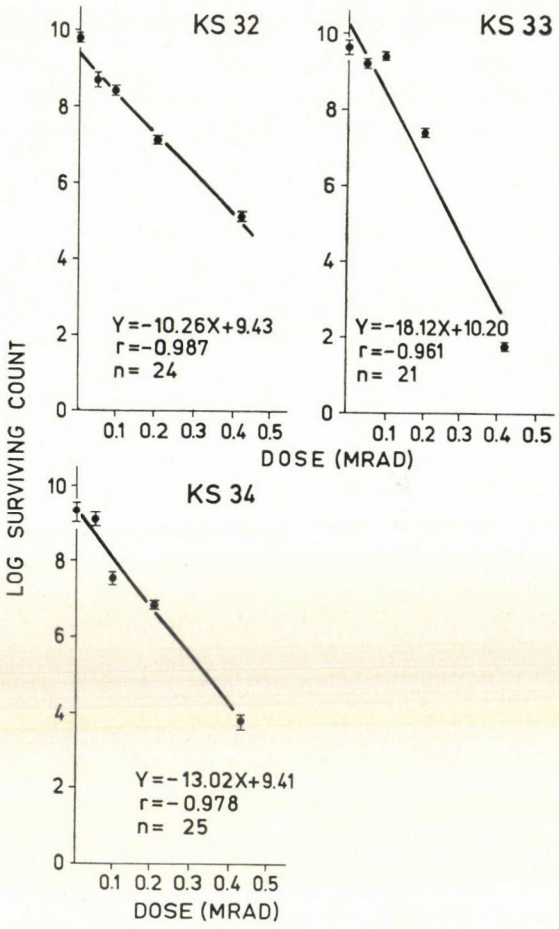

Fig. 10. The survival curves of yeasts as a function of radiation dose. (The vertical lines belonging to the mean values indicate the standard deviation)

In later stages, the consistency of the product was highly affected by the technology of freezing. Since in the course of these experiments there was no possibility of applying the proper method of freezing, only aroma and flavour were followed. It has to be noted that the quality of industrially frozen strawberries was judged significantly worse than that of the irradiated samples. It was established in the experiments that it was not possible to differentiate between yoghurt made with untreated strawberries and with strawberries irradiated with 0.57 Mrad.

The storage life of yoghurt made with strawberries depends to a large extent on the microbiological stability of the fruit. In accordance with the tests and calculations carried out, the cell count has to be below 0.01, *i.e.*, 100 g

strawberries must not contain more than 1 viable cell in order to be able to keep the yoghurt for two weeks at 2 °C.

Taking the storage time necessary to reach 20% spoilage as a measure of the relative keeping quality, in the case of yoghurt manufactured with strawberries given treatments combined with irradiation at the 0.36 Mrad level, the relative keeping quality was increased 2.6-fold at 15 °C, in comparison with yoghurt manufactured with industrially frozen strawberries, and 3.5-fold at 2 °C. The latter means the increase of the storage life from 6 days to 8 (Fig. 6).

The storability of yoghurt made with irradiated (0.57 Mrad) strawberries was only 1.5—2.0 times that of the control. It was increased from 5.6 days to 10.7 days (Fig. 8).

On studying the radiation tolerance of yeast strains isolated from strawberries it was established (pH = 5.0) that calculating with the highest D_{10} value found (D_{10} = 0.087 Mrad) and assuming an initial cell count of 10^5 g^{-1}, a minimum radiation dose of 0.435 Mrad is necessary (Figs. 9, 10). Cell counts as high as this were not found during the experiments, though spoilage was still observed. Presumably, strains more resistant than the above occur in the microbial flora of strawberries and/or the radiation resistance of yeasts on frozen strawberries is higher.

*

I would like to thank the *Ministry of Education and Science of the Netherlands*, the *International Agricultural Centre* at Wageningen and the *Institute for Cultural Relations* in Hungary for granting a fellowship to carry out this work at EURATOM-ITAL.

I am indebted to Dr. Ir. D. De Zeeuw, the Director of the Institute for arranging my work in his laboratories, enabling me to become acquainted with the work of institutes engaged in research bordering on my sphere of interest. Thanks are due to Ir. J. van Kooij and D. Is. Langerak ing. who kindly assisted in the successful accomplishment of my work and helped me with their valuable advice.

I highly appreciate the assistance given me in my work, particularly by Mr. M. F. J. Bruurs, Ir. H. Stegeman, Mr. W. A. M. Kerkhof and Mr. R. Hovestad.

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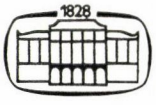

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Journal of the Food Industry Department and the National Council of Engineers and Technicians of the Socialist Republic of Roumania.

Started in 1950

Monthly issued on 64 pages

It publishes in Roumanian (with the Contents in English, French, German and Russian), original articles and condensed papers dealing with the chemistry, biochemistry and microbiology of foods and drinks; food processing, preservation, packaging and storage; the control of food products; economic problems of the food industry; technical and economic information from all over the world etc.

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Editorial Office of "INDUSTRIA ALIMENTARA" Review: Calea Victoriei 63—69, Bucharest, Roumania.

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Botyánszky Pál

A kézirat nyomdába érkezett: 1974 VIII. 30. Terjedelem: 10,5 (A/5 ív) 61 ábra

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ACADEMIAE SCIENTIARUM HUNGARICAE

A QUARTERLY OF THE COMMITTEE ON FOOD SCIENCE
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Publishing House of the Hungarian Academy of Sciences
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Acta Alimentaria, Vol. 4. (2), pp. 113–121 (1975)

DETERMINATION OF THE CAPSAICIN CONTENT OF GROUND PAPRIKA

L. ANDRÉ and L. MILE

(Received July 26, 1973)

A method was worked out for the thin-layer chromatographic determination of the capsaicin content of ground paprika with a low capsaicin content (below 100 $\mu g\ g^{-1}$).

Capsaicin was extracted with a 0.05 *N* sodium hydroxide solution containing 50% of methanol. The solutions containing the capsaicin were collected, the proteins and gel-forming substances precipitated with potassium aluminium sulphate and the precipitate separated by means of centrifuging. From the clear supernatant the capsaicin was extracted with diethylether and the extracts chromatographed on a Kieselgel GF-254 layer with diethylether as solvent. Capsaicin was developed with a 0.2% solution of 2,6-dichloroquinone chloroimide in methanol and appeared as a blue spot with an R_f-value of 0.6 . In this way even 0.1 μg of capsaicin per spot can be detected.

Pungency caused by capsaicin content is one of the characteristic components of the flavour of Hungarian ground paprika. The red paprika varieties on the market can be divided according to their pungency into two classes.

In one class are the paprikas with high capsaicin content and a predominantly pungent taste. The paprikas from the tropics and the so-called "hot" Hungarian varieties fall into this category; they have a capsaicin content higher than 500 $\mu g\ g^{-1}$. The paprikas in the other class contain no, or only very small amounts of capsaicin, they are the non-pungent or slightly pungent varieties. Since the sensing of the taste of capsaicin is highly subjective, it became necessary to assess the difference between the slightly pungent ground paprika of Hungarian taste and the non-pungent paprika by means of objective measurement.

We were, therefore, given the task of evolving a method by means of which very small capsaicin amounts can be measured, that is, its presence detected, so that the relationship between pungent and non-pungent sensation of taste and actual capsaicin content can be decided on the grounds of objective measurement.

MATHEW and co-workers (1971) published a detailed review on the chemistry, analysis and physiological effect of capsaicin. Several methods are known for the determination of high capsaicin contents of which those purifying

the paprika extract prior to chromatography (ANON, 1959, 1964) are of particular importance. Unfortunately, column chromatographic purification makes the determination highly time-consuming. The application of thin-layer chromatography will improve the process of purification.

By means of the photometric method of JENTZSCH and co-workers (1969) capsaicin can be determined down to 2 $\mu g\,ml^{-1}$, which means that the substance applied to the layer must contain at least 10 μg of capsaicin. In case of ground paprika with low capsaicin content the amount of capsaicin which can be isolated on the layer is not sufficient for photometric evaluation and the application of such large quantities of the extract to the layer causes additional difficulties. Since the detection of capsaicin on the thin-layer itself is a more sensitive procedure, thin-layer chromatography is preferable. According to RIOS and DUDEN (1971), 2 μg per spot of capsaicin can be measured without reagent on the layer at 281 nm by means of a densitometer. SPANYÁR and BLAZOVICH (1969) studied the sensitivity of thin-layer chromatographic reagents to capsaicin, and found that the potassium (hexacyanoferrate-III) + ferric chloride used by JUHÁSZ and TYIHÁK (1969) is the most sensitive reagent, by means of which even 0.1 μg of capsaicin per spot can still be detected. However, we felt that the fact that this is a general reagent which gives a blue spot, similar to that obtained with capsaicin, with the majority of reducing substances, is a considerable drawback.

JENTZSCH and co-workers (1969) suggested 2,6-dichloroquinone chloroimide for the detection of capsaicin on thin layer, with the lower limit of detectability of 0.1 μg of capsaicin per spot. This reagent has the great advantage of being more specific for capsaicin than the one suggested by JUHÁSZ and TYIHÁK (1969) and has become well known in the analysis of capsaicin. Because of the presence of a large number of interfering substances, the extraction of capsaicin from ground paprika with low capsaicin content is particularly difficult. As a rule, the solvents used for extraction of capsaicin also extract the dyestuffs of paprika.

Certain methods described in the literature use for extraction alkaline ether in methanol (ANON, 1964), ethylacetate in methanol (SUZUHI, 1957) and isopropylalcohol (RIOS & DUDEN, 1971).

JENTZSCH and co-workers (1969) found that identical quantities of capsaicin can be extracted from the same sample by methanol, chloroform and 70% ethanol, resp. Extraction with chloroform under reflux for one hour appeared to give the best results. SPANYÁR and BLAZOVICH (1969) and TIRIMANNA (1972) combined the removal of interfering substances with extraction using liquid-liquid partition. Capsaicin and the dyestuffs were extracted with diethylether, the ether was evaporated, the dry residue dissolved in alcohol and the dyestuffs extracted with petrolether. The alcohol was then distilled off from the alcoholic phase, the aqueous residue made alkaline and the cap-

saicin again extracted with diethylether. This last ether extract is then used for thin-layer chromatographic analysis.

For the determination of the capsaicin content of ground paprika with low (less than 100 $\mu g\ g^{-1}$) capsaicin content, of the above mentioned methods alone that of SPANYÁR and BLAZOVICH (1969) can be used. This method, too, has certain drawbacks which complicate the evaluation of the results, such as the concomitant extraction of capsaicin and of part of the dyestuffs which will react on the layer with the developing reagent. We aimed at finding an extraction method by means of which capsaicin can be separated as much as possible from the interfering substances and to apply a layer chromatographic developing reagent particularly sensitive to capsaicin.

We shall compare the method worked out by us with that of SPANYÁR and BLAZOVICH (1969).

1. Materials and methods

1.1. Materials and equipment

Diethylether, peroxide free, analytical grade.
Methanol, analytical grade.
Acetone, analytical grade.
Capsaicin, analytical grade.
2,6-dichloroquinone chloroimide, analytical grade.
Sodium hydroxide, analytical grade.
Potassium aluminium sulphate, analytical grade.
Hydrochloric acid, analytical grade.
Kieselgel GF-254.
Centrifuge, with stoppered tubes, JANETZKI T 30 typ. 4.5×10^3 g.
Shaker, LABOR type LE 203.
Drying cabinet, LABOR LP-301.
Thin-layer chromatographic set, DESAGA.
Micropipette, HAMILTON 5 μl; 25 μl.
Spectrophotometer, SPECORD UV—VIS.

1.2. Extraction of capsaicin from ground paprika

About 2 g of ground paprika is weighed to the nearest centigram and extracted, in a stoppered centrifuge tube, on the shaker set at grade 1 with 3×20 ml of 0.05 N sodium hydroxide containing 50% of methanol for 10 minutes each time. The ground paprika is separated after each extraction from the alkaline methanol extract by centrifuging with 4.5×10^3 g for 10 minutes. The extracts are collected in flasks containing 0.5 g of potassium

aluminium sulphate and 7 ml of 0.5 N hydrochloric acid. After the addition of the last extract the mixture is thoroughly shaken, allowed to stand for 10 minutes during which period it is again thoroughly shaken every two minutes. A yellowish red precipitate is formed which is separated by centrifuging. The clear solution is poured into a separating funnel and the centrifuged precipitate washed with 0.001 N hydrochloric acid containing 50% of methanol and again centrifuged. The clear supernatant is also poured into a separating funnel and extracted with 3×20 ml of peroxide-free diethylether. The sharply separated diethylether layers are collected after filtration through anhydrous sodium sulphate. The volume of the ether phase is evaporated to 10 ml on a 40 °C waterbath in air stream.

1.3. Thin-layer chromatography

The chromatogram is run on a 20×20 cm, 0.2 mm thick Kieselgel GF-254 layer which has been activated at 105 °C for 30 minutes. Depending on the capsaicin content, 0.05 to 0.15 ml of the ether extract is applied to the layer.

Beside the solution with unknown capsaicin content, 0.1 μg to 0.5 μg portions of capsaicin are applied as standard to the layer. The chromatogram is run at room temperature, using diethylether as solvent, to a length of 15 cm, dried in hot air and developed at room temperature with a 0.2% solution of 2,6-dichloroquinone chloroimide in methanol. The dried plate is sprayed evenly with the developing solution, dried again, sprayed with a 0.1 N solution of sodium hydroxide and dried. The blue spots characteristic of capsaicin appear within a few seconds at $R_f = 0.6$.

The blue capsaicin spots are stable for 3 to 4 hours. Quantitative evaluation is based on the size of the spots and the intensity of their colour by visual comparison to the size and intensity of the spots of known amounts of capsaicin.

2. Results

We have compared the results of our extraction and chromatographic method to those obtained by the thin-layer chromatography of SPANYÁR and BLAZOVICH (1969). We determined the capsaicin content of three ground paprika samples marketed under different names and complying with the specifications of the Hungarian Standard. Five parallel determinations were carried out by both methods.

Table 1 contains the analytical data of the ground paprika of "sweet" quality, including the capsaicin content and the pertaining standard deviations. The two methods were compared by means of the t and F tests whose results are also given in the table.

Table 1

Analytical data of "sweet" ground paprika complying with the specifications of the Hungarian Standard

	Capsaicin content ($\mu g\ g^{-1}$)	Deviation from the average ($\mu g\ g^{-1}$)	Standard deviation ($\mu g\ g^{-1}$)	t	F
Determination according to the method of SPANYÁR and BLAZOVICH (1969)	9	0	1.58	0.368	1.265
	11	2			
	8	1			
	7	2			
	8	1			
average	9				
Determination according to the method of the authors	9	1	2.00		
	9	1			
	8	2			
	11	1			
	13	3			
average	10				

The analytical data of "dessert" quality ground paprika are given in Table 2, and those of the "semi-sweet" quality in Table 3.

3. Conclusions

Capsaicin was extracted from ground paprika so that the presence of pigments and other interfering substances in the solution to be subjected to capsaicin detection can be avoided as completely as possible. The alkaline solution of 50% methanol was found the most suitable for this purpose, since this methanol concentration is not high enough for the extraction of the majority of pigments and only the non-esterified, oxidized derivatives of the carotenoids will go into solution. Capsaicin can be extracted from ground paprika by three subsequent extractions. The extract is in the colloidal state which interferes with extraction by diethylether in which an emulsion is formed. This necessitated the clarification of the methanol solution which was performed after neutralization with potassium aluminium sulphate, *i.e.* with the aluminium hydroxide precipitate formed. In this way the quantity of dyestuffs and of other interfering substances was considerably reduced in the solution. The difference between the method suggested by us and that used by SPANYÁR and BLAZOVICH is illustrated in Fig. 1.

Fig. 2 shows the chromatogram obtained by the method of SPANYÁR and BLAZOVICH (1969).

Table 2

Analytical data of "dessert" quality ground paprika complying with the specifications of the Hungarian Standard

	Capsaicin content ($\mu g\ g^{-1}$)	Deviation from the average ($\mu g\ g^{-1}$)	Standard deviation ($\mu g\ g^{-1}$)	t	F
Determination according to the method of SPANYÁR and BLAZOVICH (1969)	10	2			
	6	2			
	7	1	1.87		
	10	2			
	9	1			
average	8			0.958	1.326
Determination according to the method of the authors	10	1			
	8	1			
	8	1	1.41		
	7	2			
	10	1			
average	9				

Table 3

Analytical data of "semi-sweet" quality ground paprika complying with the specification of the Hungarian Standard

	Capsaicin content ($\mu g\ g^{-1}$)	Deviation from the average ($\mu g\ g^{-1}$)	Standard deviation ($\mu g\ g^{-1}$)	t	F
Determination according to the method of SPANYÁR and BLAZOVICH (1969)	27	2			
	26	3			
	28	1	2.73		
	33	4			
	29	0			
average	29			0.000	1.241
Determination according to the method of the authors	29	0			
	25	4			
	34	5	3.39		
	27	2			
	28	1			
average	29				

Column A shows the chromatogram prior to developing, column B is the chromatogram developed with potassium (hexacyanoferrate-III) + ferric chloride reagent.

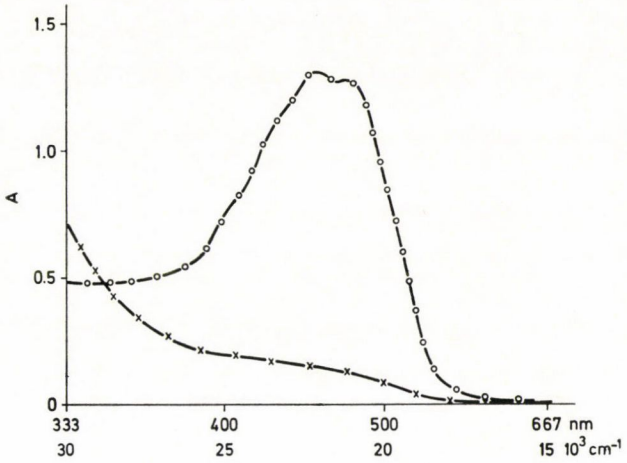

Fig. 1. Spectra of ground paprika extracts from the same sample. —o—o— extract prepared according to the method of SPANYÁR and BLAZOVICH (1969); —x—x— extract prepared according to the method suggested by the authors. The capsaicin concentration is the same in the two extracts. The spectra were recorded with the spectrophotometer *Specord* using a 1-cm cell

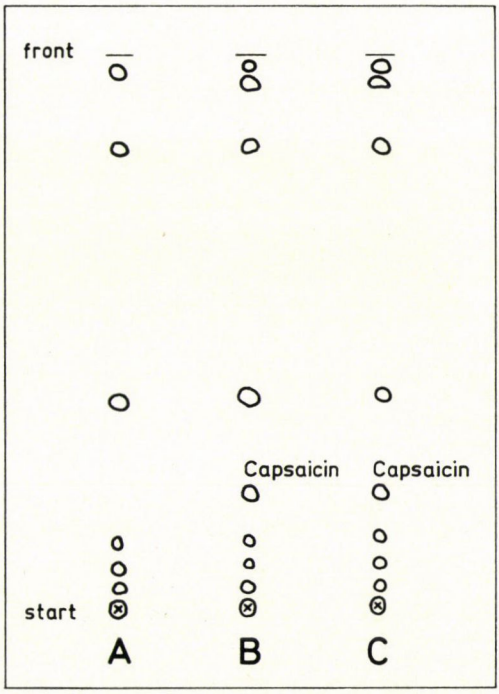

Fig. 2. Chromatogram of the extract prepared according to the method of SPANYÁR and BLAZOVICH (1969). *A* — chromatogram prior to developing; *B* — chromatogram developed with potassium (hexacyanoferrate-III)+ ferric chloride; *C* — chromatogram developed with 2,6-dichloroquinone chloroimide

All spots are blue, including that of capsaicin.

Column *C* is the chromatogram developed with 2,6-dichloroquinone chloroimide. The carotenoids are not discoloured, they have kept their initial colour but decompose on air after some time. There is a grey spot at the starting point caused by some product of phenolic type.

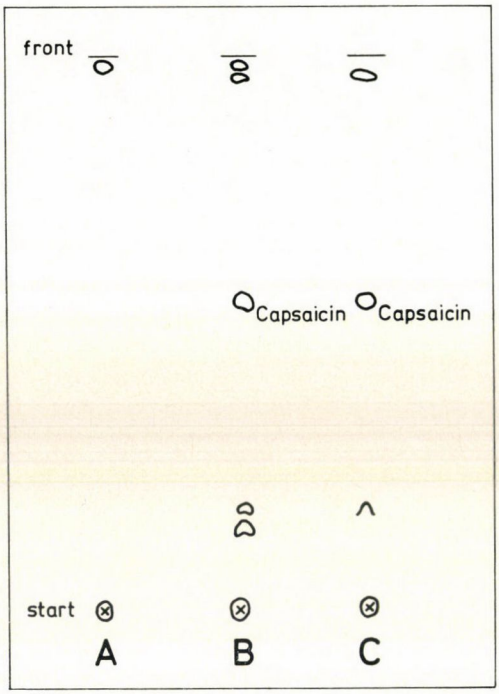

Fig. 3. Chromatogram of the extract prepared according to the method suggested by the authors. *A* — chromatogram prior to development; *B* — chromatogram developed with potassium (hexacyanoferrate-III)+ferric chloride reagent; *C* — chromatogram developed with 2,6-dichloroquinone chloroimide

The chromatogram of the extract prepared by our method is shown in Fig. 3.

Column *A* is the chromatogram prior to developing; the alkaline solution of 50% methanol removes the carotene from the ground paprika, as indicated by two coloured spots. Column *B* is the chromatogram after it has been developed with potassium (hexacyanoferrate-III) + ferric chloride; it appears that the reducing substance not investigated by us has also been extracted from the ground paprika. Column *C* is the chromatogram developed with 2,6-dichloroquinone chloroimide.

Comparison of columns *B* in Fig. 2 and of *C* in Fig. 3 shows the difference between the method of SPANYÁR and BLAZOVICH and the method suggested by us.

Statistical analysis showed no significant difference between the results obtained by the two methods.

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We wish to thank the Director of the Institute, Mr. GY. HORVÁTH for creating the conditions for the elaboration of our subject in our Institute and also for the attention with which he followed our work and helped it with his valuable advice.

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Acta Alimentaria, Vol. 4. (2), pp. 123—138 (1975)

THE INFLUENCE OF IRRADIATION AND PACKAGING ON THE KEEPING QUALITY OF PREPACKED CUT ENDIVE, CHICORY AND ONIONS

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(Received September 11, 1973)

The quality of prepacked endive, chicory and onions deteriorates very quickly by discolouration, desiccation and microbiological spoilage.

Because cooling at about 10 °C during transport and sale did not give satisfactory results, the influence of irradiation on the keeping quality has been investigated.

The experiments with endive and chicory showed that by washing immediately after cutting, pink-discolouration decreased. During storage, however, this discolouration increased again.

Discolouration was partially prevented by packaging in non-perforated polythene foil of 0.02 mm thereby decreasing the O_2 and increasing the CO_2 content in the bags.

A difficulty in using non-perforated bags was that in non-irradiated products, fermentation took place caused by a high microbial count (10^8 g^{-1}). In irradiated samples no fermentation occurred probably due to the decreased microbial count.

Cut onions had to be packed in perforated polythene bags because strong gas (CO_2) development took place in non-perforated bags at temperatures above 10 °C.

The initial total viable count of endive, chicory and onions varied from 10^3 to 10^7 g^{-1}. Irradiation with doses above 50 krad gave a reduction of this count of 3—6 log cycles through which the keeping time was lengthened by about 100% at 10—20 °C.

The *Enterobacteriaceae* were mainly eliminated by irradiation doses higher than 100 krad.

The optimal dose for chicory, onions and endive was found to be 50, 75, and 100 krad, resp. Higher doses intensified discolouration and influenced unfavourably the flavour.

Cooking experiments with cut onions packed in perforated bags showed that the non-irradiated product discoloured quickly into brown after cooking. By irradiation with 75—100 krad this discolouration was prevented.

The need for prepackaged and pretreated horticultural products is increasing as a consequence of the development of the self-service system in shops and supermarkets and because of the lack of personnel in factory canteens and at large institutions *e.g.* hospitals and homes for the aged. Hitherto prepackaging took place at the self-service shops and supermarkets themselves.

In the last few years there is a clear tendency to carry out the prepackaging centrally to achieve more economical and efficient use of labour and equipment. However, centralization makes heavy demands on the keeping quality during storage, transport and sale.

The main problems of prepacked cut vegetables are:

a) discolouration, b) microbiological spoilage.

The discolouration is probably caused by:

- an oxidation of the phenolic substances available in the cell sap, catalyzed by such enzymes as polyphenoloxidase or tyrosinase;
- a non-enzymatic reaction through a direct oxidation or polymerization of polyphenols. [MAXIE and ABDAL-KADER (1966), MATHEW and PARPIA (1971].

In both reactions O_2 plays an important role.

Spoilage can be delayed for a couple of days by cooling. A better method is vacuum cooling. The latter system demands perforations in the package for a quick cold transfer. However, perforations are unfavourable for the colour, because these holes cause a high O_2 content in the package.

Another problem of cooling is the maintenance of a complete cold-chain during transport and sale. Practice has shown that this is very expensive and often difficult to maintain. The shelf-life of prepacked vegetables is also 1 to 2 days which is too short for centralization.

Because under practical conditions cooling alone did not give the desired results, the application of ionizing radiations has been studied. Experiments have been carried out with cut endive, cut onions and cut chicory.

1. Materials and methods

The products were cut mechanically in strips of 5—8 mm thickness with a GLASTRA vegetable cutting apparatus, type *Ideaal* and washed in tap-water in a GLASTRA vegetable washing apparatus, type *Kristal* for 1.5 min for removing the soil particles.

After washing the product was centrifuged for about 1.5 min with a spin-drier (900 *g*) for vegetables (FAM. CHAMPION type FM 11) in order to remove the extra washing water. An amount of washing water higher than 3—5% influenced the storability unfavourably by causing decay.

After packaging the irradiation was carried out at the Pilot Plant for Food Irradiation at Wageningen (^{60}Co source of 105 kCi, dose rate 1.4 krad min^{-1}, 10% accuracy) 1—5 hours after handling.

During storage of the prepacked vegetables the following criteria were evaluated:

- Quality (colour, desiccation and visible decay).
  The product was given scores 5 to 1; 5 denoting optimal and 1 very bad condition.

— Gas composition in the bags by measuring 3 bags of each product by means of an *Orsat* apparatus.
— Organoleptic tests on the cooked product. The quality was assessed by a lab/consumer panel. A hedonic scale 1—10 was used (1—2 bad, 3—4 objectionable, 5 limit of acceptability, 6 medium, 7—8 good, 9—10 excellent). Test data were calculated according to the Quick Rank Test of KRAMER (1956, 1960).
— Microbiological contamination. The total viable count was determined on a Plate Count Agar (P.C.A.) medium (OXOID CM325) after 2 days incubation at 35 °C. For the detection and enumeration of *Enterobacteriaceae* a Violet-Red-Bile-Glucose (V.R.B.) medium (OXOID CM323) was used. The *Enterobacteriaceae* were incubated 24 hours at 30 °C. Of each product 3 bags were examined. Determinations were carried out in duplicate.

The design per product follows below.

1.1. Endive (Cichorium endivia L.)

The experiments have been carried out with outdoor and greenhouse endive of different varieties. For each experiment about 30 kg fresh endive were used.

After cutting in strips of 5—8 mm, washing and centrifuging for 1.5 min, resp. the product was packed in polythene bags (content 500 g) of 0.02 and 0.05 mm thickness without and with 2 perforations of 2 mm diameter.

The doses applied were 0, 100, 150, 250 and 300 krad, resp. The product was stored at 2 °, 10 ° and 12 °C, resp. (LANGERAK *et al.*, 1972).

1.2. Chicory (Cichorium intybus L.)

The product came from a retail trader, an auction or a grower. All samples were handled in the same way as endive only the spin-drier time after washing was varied from 1/4 to 1 minute.

As an anti-oxidant cystein was used in concentrations of $10^{-4}M$, $5\times10^{-4}M$, $10^{-3}M$ and $5\times10^{-3}M$.

The product was packed in bags of: a) polythene of 0.02 mm thickness without and with 2 perforations of 2 mm diameter, b) polythene of 0.05 mm thickness without perforations.

The irradiation doses applied were 0, 50, 100, and 150 krad, respectively. The product was stored at about. 10 °C (8 hours light, 16 hours darkness) (LANGERAK & HOVESTAD, 1973).

1.3. Onions (Allium cepa L.)

The first experiments were carried out under laboratory conditions. The onions were cut mechanically in 3 mm thick slices. The product was not washed, because washing removes the specific onion odour.

The product was packed in polythene bags of 0.02 mm thickness, without perforations, irradiated with 0, 50, 100 and 150 krad gamma radiation and stored at 15 °C.

The other experiments were carried out under commercial conditions. The product was packed in polythene bags of 0.02 mm and 0.065 mm thickness provided with 2 perforations of 2 mm diameter.

The irradiation doses were 0, 75 and 100 krad, respectively (LANGERAK & HOVESTAD, 1972).

2. Results

2.1. Endive

The experiments with endive proved that quality retention during storage depended to a great extent on variety and initial qualities. The fresh

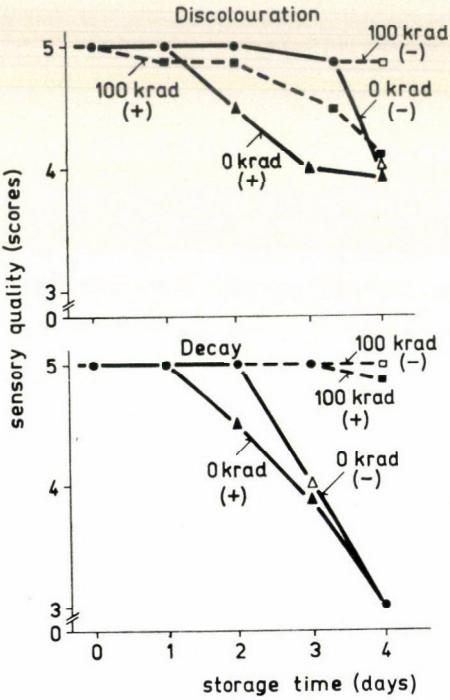

Fig. 1. Influence of irradiation and packaging on the discolouration and visible decay of prepacked cut endive during storage at 12 °C. The endive was packed in perforated (+) and non-perforated (−) polythene bags of 0.02 mm thickness. • denotes coinciding experimental points pertaining to two or more treatments

product discoloured less than the desiccated one. Further the discolouration was mostly more pronounced in outdoor endive than in greenhouse endive, probably due to a higher percentage of white leaves in the outdoor endive.

The pink-brown discolouration of the cutting surface of the white part of the leaves was one of the main problems in the keeping quality of the prepackaged cut endive.

At the beginning of storage, samples irradiated with a dose higher than 150 krad had a little more discolouration than the non-irradiated samples. But during extended storage the discolouration of the non-irradiated samples increased whereas in the irradiated samples the colour remained almost unchanged. At the beginning of storage a dose of 100 krad did not cause significantly more discolouration than was observed in the non-irradiated samples (Fig. 1).

Besides by the irradiation dose, the pink-brown discolouration was also influenced by the packaging. Packaging in sealed polythene bags resulted in a modified gas atmosphere in the bags. During storage in the non-perforated bags the O_2 content fell and the CO_2 content rose considerably, thus, partially preventing discolouration.

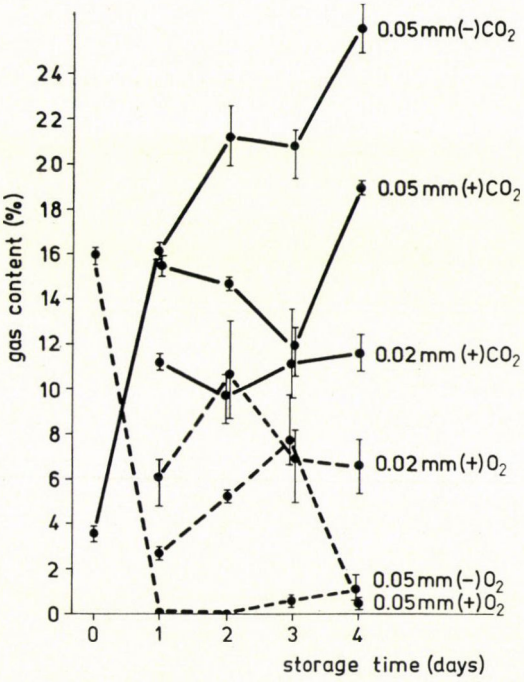

Fig. 2. Composition of the atmosphere in packs of cut endive. The average gas percentages with the highest and lowest values measured in 3 non-irradiated bags. The cut endive was packed in perforated (+) and non-perforated (—) polythene bags of 0.02 and 0.05 mm thickness and stored at 12 °C

However, an O_2 content $< 1\%$ and a CO_2 content $> 10\%$, led to intermolecular respiration and souring (fermentation), especially in non-irradiated bags in which the microbial count increased very quickly (10^8 g^{-1}).

Gas measurements in polythene bags of 0.05 mm thickness showed an unfavourable gas composition, because the foil was too impermeable (Fig. 2).

Perforation increased the O_2 content and decreased the CO_2 content; a high O_2 content, however, increased the discolouration probably owing to increased oxidation of the polyphenols.

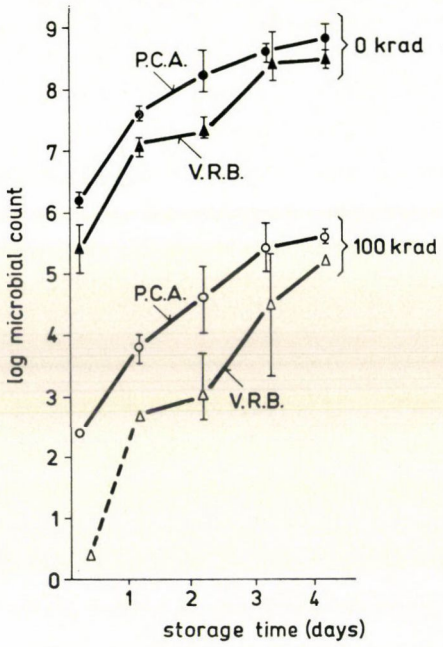

Fig. 3. Microbiological changes in packaged cut endive caused by irradiation. Average log count of 3 samples with the highest and lowest values, determined on P.C.A.-medium (total viable count) and V.R.B. medium *(Enterobacteriaceae)*. The endive was stored in non-perforated polythene bags (0.02 mm) at 12 °C

As regards quality (colour and decay), optimal results were obtained with a dose of 100 krad in combination with a non-perforated bag of 0.02 mm thickness.

Microbiological studies proved that the initial total viable count of cut endive was about 10^6 g^{-1}. This count increased to 10^8 g^{-1} in a couple of days at temperatures above 10 °C. Visible decay was detected when the total viable count exceeded 10^7 g^{-1}. An irradiation of 100 krad gave a reduction of 4 to 5 log cycles (Fig. 3). Consequently the spoilage was strongly delayed and the shelf-life was lengthened by about 100%.

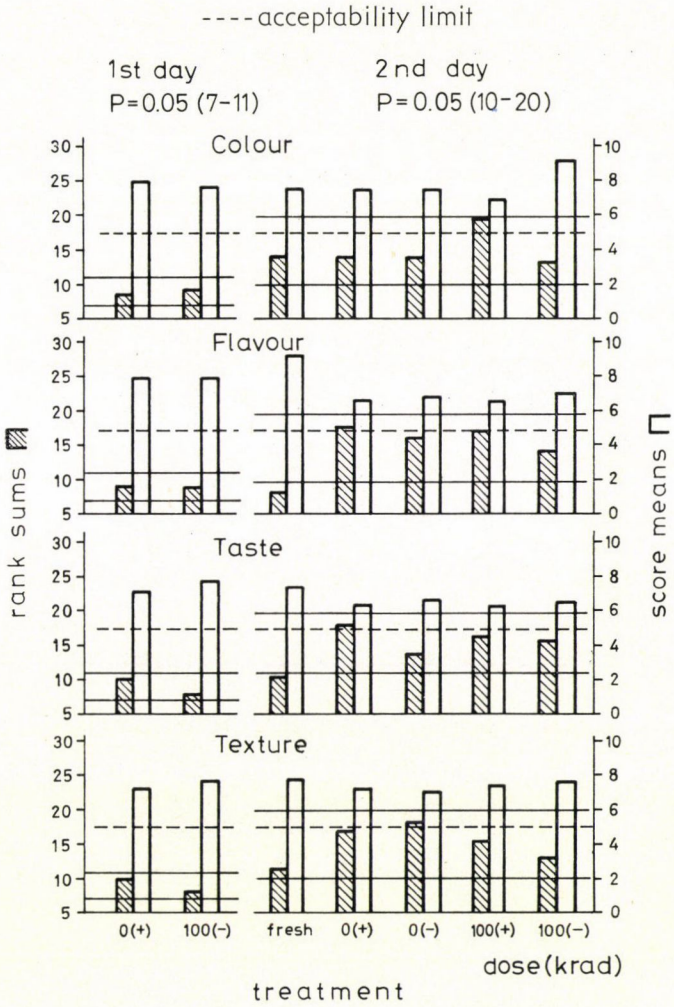

Fig. 4. Sensory quality of irradiated packaged cut endive in the cooked state. Results of the organoleptic tests of cut endive packaged in perforated (+) and non-perforated (—) polythene bags (0.02 mm), stored for 1 and 2 days, resp. at 12 °C. Differences in rank sums between the lowest and highest limits (7—11) and (10—20) are not significant at $P = 0.05$. The acceptability limit of the score means is 5. Number of panelists: 6 and 5, resp.

The initial count of *Enterobacteriaceae* was about 10^5 g^{-1}. During storage at temperatures above 10 °C the number of *Enterobacteriaceae* in the non-irradiated samples went up to 10^7 g^{-1} after 1 to 2 days, whereas in irradiated samples it was mostly less than 10^3 g^{-1}.

The increase in microbial count at 2 °C was very slow, so that the difference in keeping quality between the irradiated and the non-irradiated samples was very small. Sensory tests of the *cooked* product proved that irradiation of

prepacked cut endive with doses higher than 150 krad resulted in off-flavour and objectionable discolouration. At doses of 100—150 krad the irradiated samples did not differ from the control during the first days after irradiation;

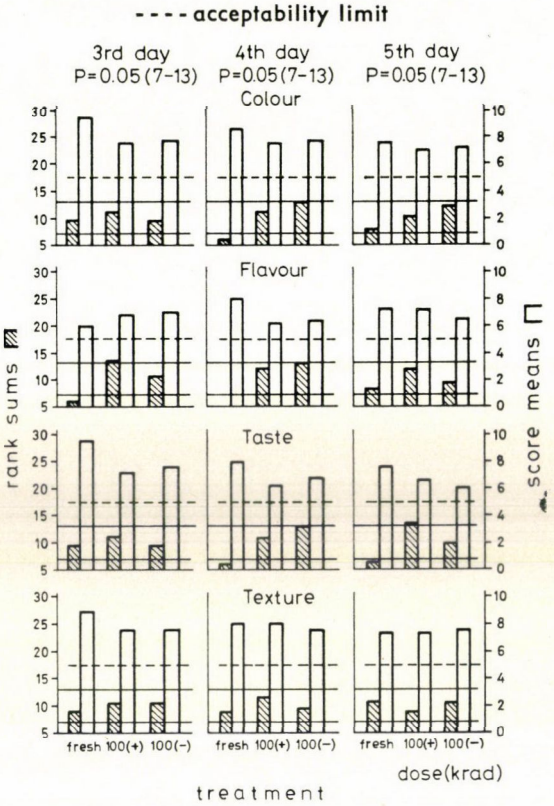

Fig. 5. Sensory quality of irradiated packaged cut endive in the cooked state. Results of the organoleptic tests of cut endive packed in perforated (+) and non-perforated (—) polythene bags (0.02 mm), stored for 3 and 5 days resp. at 12 °C. Differences in rank sums between the lowest and highest limits (7—13) are not significant at P = 0.05. The acceptability limit of the score means is 5. Number of panelists: 5

later on they were found in some experiments to be better than the non-irradiated samples (Figs. 4 and 5).

2.2. Chicory

The experiments with chicory showed that by washing immediately after cutting pink discolouration decreased. During storage, however, the

colour change increased again. The influence of centrifuging time on colour was small (Fig. 7); only at 1/4 min centrifuging somewhat less drying took place than at 1 min.

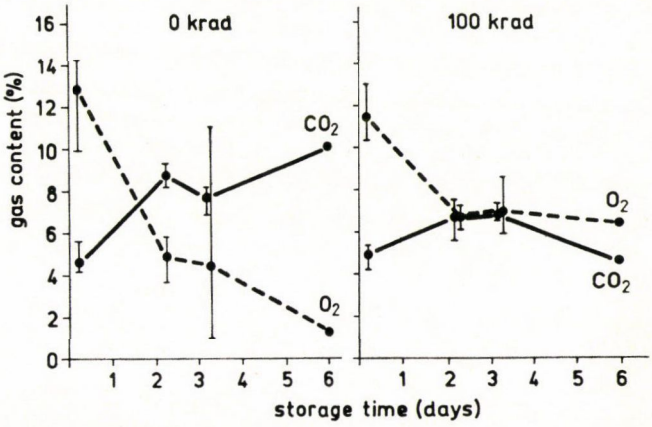

Fig. 6. Effect of irradiation on the composition of the atmosphere in packs of cut chicory. The average gas percentages with the highest and lowest values measured in 3 bags. The cut chicory was packed in non-perforated polythene bags of 0.02 mm thickness and stored at 10 °C

In the perforated bags the chicory discoloured considerably quicker than in the non-perforated bags. A film thickness of 0.02 mm was better than 0.05 mm because with the latter the CO_2 content in the bags rose above 10%.

A difficulty in using non-perforated bags was that in non-irradiated samples fermentation could take place as a consequence of the low O_2 content ($< 1\%$), and the high CO_2 content ($> 10\%$) (Fig. 6) as well as the high viable count $10^8\ g^{-1}$. In irradiated samples no fermentation occurred probably due to the lower viable count.

At the beginning of storage the pink-discolouration was slightly intensified by irradiation. This discolouration was greater at higher doses. During storage of irradiated lots, however, discolouration remained stable, whereas in non-irradiated samples discolouration worsened probably due to decay as well (Fig. 7).

In non-perforated packaging, irradiation gave no obvious improvement with regard to colour. In perforated bags, however, less discolouration took place in irradiated samples than in non-irradiated ones (Fig. 8).

The initial total viable count of chicory was found $10^5\ g^{-1}$ (Table 1). After 2 to 3 days storage at 10 °C it had risen to $10^8\ g^{-1}$. Irradiation with doses higher than 50 krad gave a reduction of 3—4 orders of magnitude which lengthened the keeping quality by 100%.

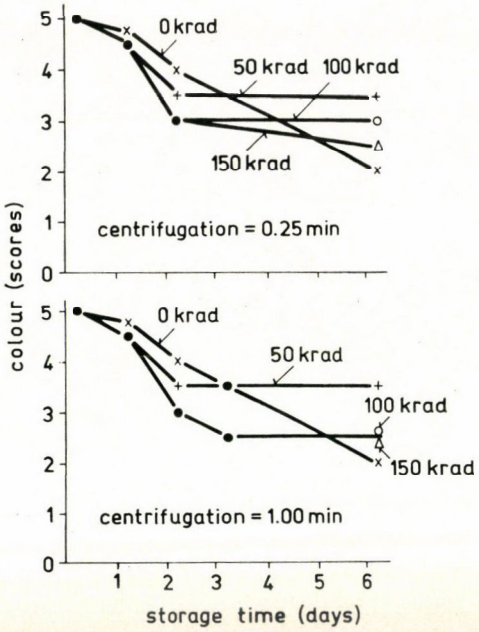

Fig. 7. Influence of irradiation and centrifugation time on discolouration of cut chicory. The product was packaged in non-perforated polythene bags of 0.02 mm thickness and stored at 10 °C. • indicates coinciding experimental points pertaining to two or more treatments

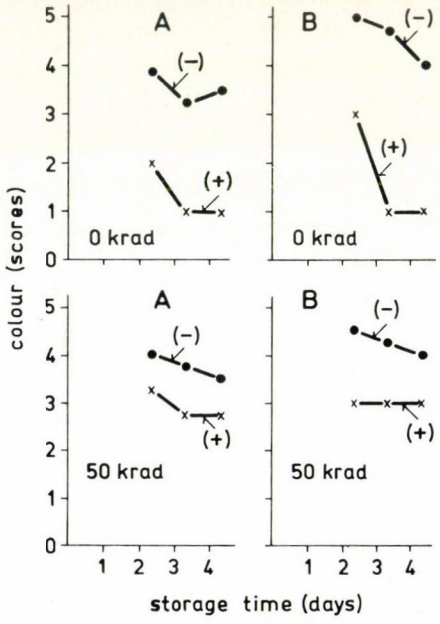

Fig. 8. Influence of irradiation, packaging and addition of cystein ($10^{-3}M$) on the discolouration of cut chicory. The product was packaged in non-perforated (−) and perforated (+) polythene bags of 0.02 mm thickness and stored at 10 °C. (A) — washed in water; (B) — dipped in cystein

Table 1

Influence of irradiation on the total viable count

Days	Total viable count (g^{-1}) in prepacked cut chicory stored at 10 °C			
	krad			
	0	50	100	150
	3.0×10^5	60	80	<50
0	1.4×10^5	90	70	<50
mean	2.2×10^5	75	75	<50
	1.9×10^5	1.4×10^2	1.0×10^2	—
1	3.1×10^5	1.5×10^2	70	—
mean	2.5×10^5	1.4×10^2	85	—
	1.2×10^7	5.5×10^3	<50	—
2	1.4×10^7	5.5×10^3	<50	—
mean	1.3×10^7	5.5×10^3	<50	—
	1.0×10^8	1.0×10^4	6.5×10^2	1.2×10^2
3	0.6×10^8	0.9×10^4	3.0×10^2	3.0×10^2
mean	0.8×10^8	1.0×10^4	4.8×10^2	2.1×10^2

The influence on the colour of the 4 different cystein concentrations varied (Fig. 8). There was no preference for any of the 4 concentrations. In non-perforated bags decay even tended to increase by cystein treatment.

From preliminary cooking experiments it appeared that no great differences were brought about by the different treatments.

2.3. Onions

Laboratory experiments showed that non-perforated packaging was favourable in regard to colour.

The preservation of colour probably was due to the low O_2 content of 1—4% and the high CO_2 content of 7—10% (Fig. 9).

However, in non-irradiated onions fermentation quickly took place due to the high total viable count ($10^8\,g^{-1}$), the low O_2 content ($< 1\%$) and the high CO_2 content ($> 10\%$).

In the irradiated onion fermentation hardly occurred because here the microbial count was considerably lower, which favourably influenced the composition of the atmosphere in the bags. In the non-perforated bags no great differences occurred in colour between the irradiated and the non-irradiated samples during the first 3 days of the storage at 15 °C. However, after the 4th day the colour of the irradiated product showed less decay.

The initial total viable count in the P. C. A. medium was $10^3\,g^{-1}$, which shows that the product was very "clean". Nevertheless after 3 days this count increased to $> 10^8\,g^{-1}$ (Fig. 10).

Irradiation brought about a distinct reduction of more than 3 orders of magnitude. In spite of this reduction, the total count strongly increased also in the irradiated product. So it appeared that cut onions are very perishable.

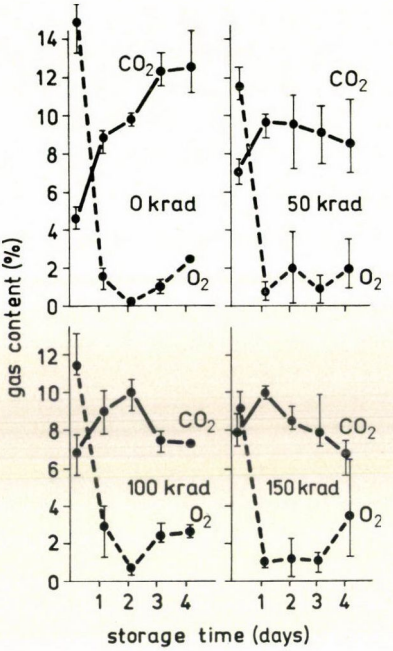

Fig. 9. Effect of irradiation on the composition of the atmosphere in packs of cut onions. The average gas percentages with the highest and lowest values measured in 3 samples. The cut onions were packaged in non-perforated polythene bags of 0.02 mm thickness, irradiated with different doses and stored at 15 °C

In the non-irradiated products the count of *Enterobacteriaceae* strongly increased. After 4 days storage it rose from $< 10^2\,g^{-1}$ to almost $10^8\,g^{-1}$ and irradiation with 100 krad brought about a distinct reduction.

Experiments under practical conditions showed that the product discoloured quicker in perforated than in non-perforated bags. After 1 day discolouration in the non-irradiated product was worse than in the irradiated samples, probably as a result of spoilage.

The microbial count of the product cut under commercial conditions was $10^5 - 10^8\,g^{-1}$, *i.e.* more than 3 orders of magnitude higher than when the product was handled under laboratory conditions. The non-irradiated product spoiled already after 2 days ($10^8 - 10^9\,g^{-1}$).

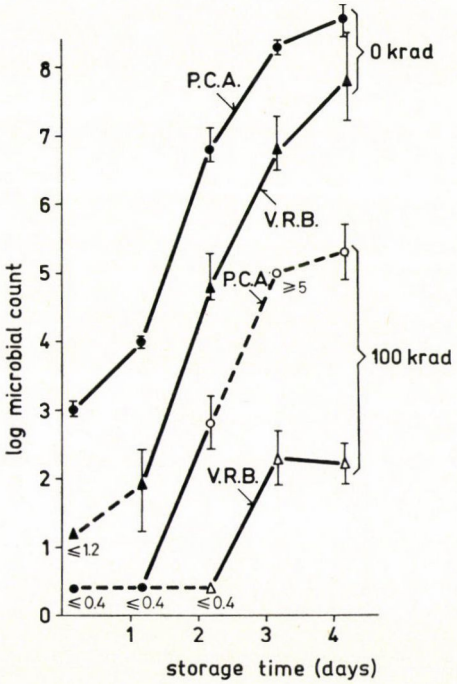

Fig. 10. Effect of irradiation on microbiological changes in packaged cut onions during storage. Average log counts of 3 samples of cut onions with the highest and lowest values as determined on P.C.A.-medium (total viable count) and V.R.B. medium *(Enterobacteriaceae)*. The onions were stored in non-perforated polythene bags (0.02 mm) at 15 °C

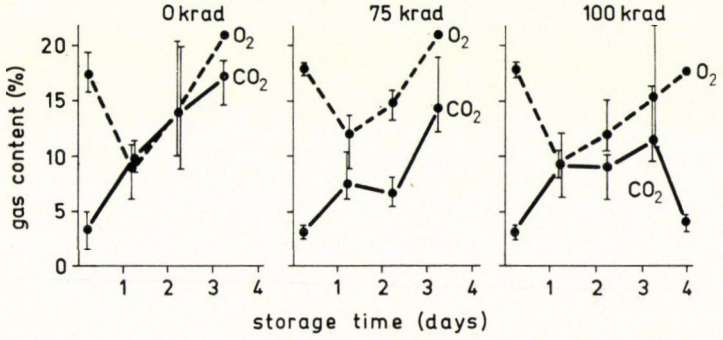

Fig. 11. Effect of irradiation on the composition of the atmosphere in packs of cut onions. The average gas percentages with the highest and lowest values, measured in 3 samples. The cut onions were packaged in perforated polythene bags of 0.02 mm thickness and stored at 15 °C

Irradiation with 75 and 100 krad brought about a reduction of 3 and 4 log cycles, respectively. In spite of this reduction, the keeping quality increased by only 1 or 2 days. Also the initial count of *Enterobacteriaceae* was high in

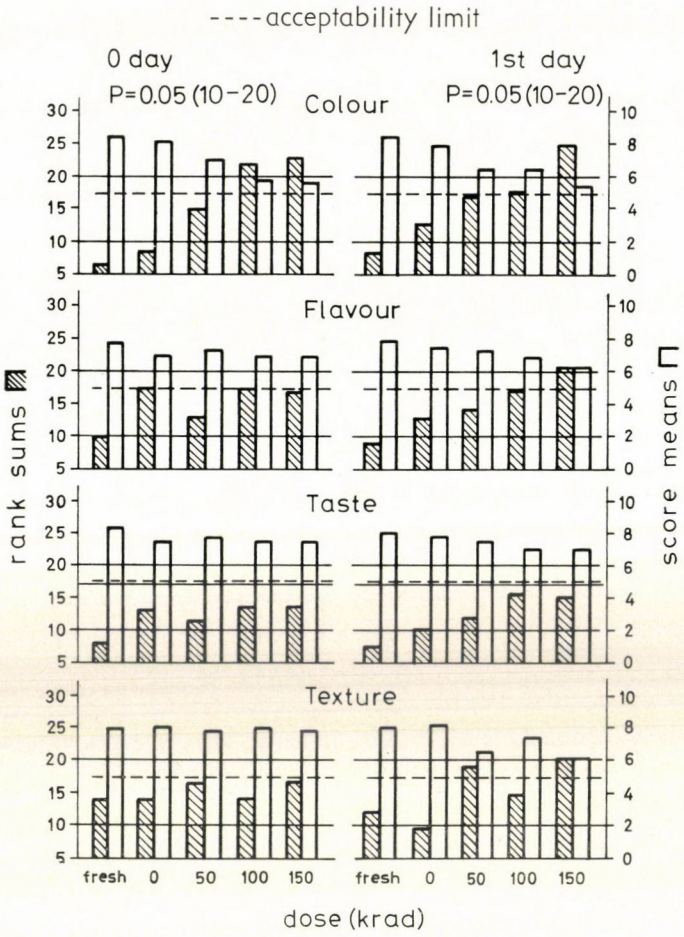

Fig. 12. Sensory quality of irradiated packaged cut onions in the cooked state. Results of the organoleptic tests of cut onions packaged in non-perforated polythene bags (0.02 mm), stored for 0 and 1 day at 15 °C. Differences in rank sums between the lowest and highest limits (10—20) are not significant at P = 0.05. The acceptability limit of the score means is 5. Number of panelists: 5

these experiments (10^6 g^{-1}). Irradiation brought about a reduction of 4—5 orders of magnitude, which was not sufficient to prevent growth completely.

In the 0.065-mm polythene bags, the CO_2 content surpassed 10%, in spite of the perforations. As a result of the perforation, the O_2 content was fluctuating around 10% (exception: the 0-krad sample). Also in the perforated polythene bags of 0.02 mm, a strong development of gas took place (Fig. 11). The increase of CO_2 content was parallel to the strongly increased total viable count.

The results of sensory tests of cooked prepacked cut onions that had been stored in perforated polythene bags are reproduced in Figs. 12 and 13. Non-

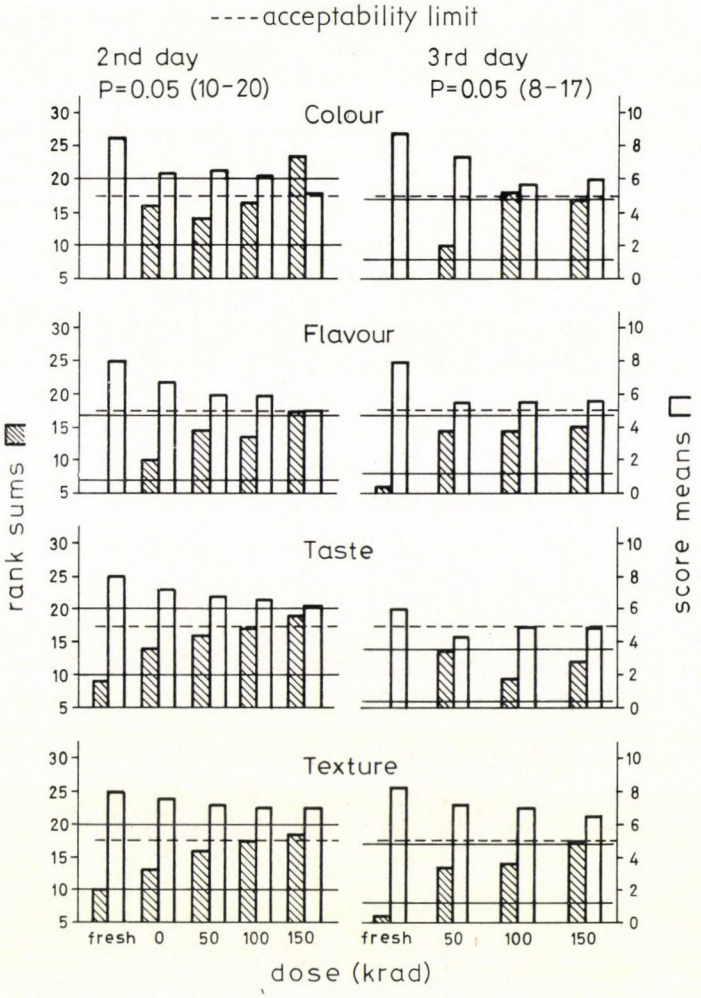

Fig. 13. Sensory quality of irradiated packaged cut onions in the cooked state. Results of the organoleptic tests of cut onions packaged in non-perforated polythene bags (0.02 mm), stored for 2 and 3 days resp., at 15 °C. Differences in rank sums between the lowest and highest limits (10—20) and (8—17) are not significant at P = 0.05. The acceptability limit of the score means is 5. Number of panelists: 5

perforated packaging was favourable with regard to the colour of the cooked product; no browning occurred.

In general flavour and colour of the cooked product were hardly affected by irradiation. However, with doses higher than 100 krad a slight grey discolouration occurred. Perforated packing was unfavourable with regard to the colour of the cooked product.

Cooking experiments showed that the non-irradiated product became brown and was not acceptable. During storage this discolouration into brown

grew more and more intensive. The irradiated product on the contrary remained practically white; only in the 75-krad sample did some discolouration occur after 3 days.

3. Conclusions

The experiments showed that the application of gamma rays for the centralization of prepackaged cut vegetables offers perspectives, provided the following conditions are taken into account:

- As soon as possible after cutting, the product has to be washed (except onions), centrifuged and packed in non-perforated polythene bags of 0.02 mm thickness. However, onions have to be packed in perforated bags, because strong gas (CO_2) development takes place during storage at temperatures around 10 °C.
- In combination with the packing mentioned, irradiation is necessary to prevent fermentation and decay. The keeping time is lengthened hereby by about 100% at 10—20 °C.
- The optimum radiation dose for chicory, onions and endive is 50, 75 and 100 krad, respectively. Higher doses intensify discolouration at the beginning of storage.

Furthermore, from these experiments it appeared that irradiation increases the hygienic quality by a reduction of the initial count of *Enterobacteriaceae*.

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Acta Alimentaria, Vol. 4. (2), pp. 139—150 (1975)

CHANGES IN THE TRIGLYCERIDE STRUCTURE DURING THE HARDENING OF SUNFLOWER SEED OIL

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(Received December 3, 1973)

The distribution of triglyceride groups containing fatty acid components of various degrees of saturation was studied in natural sunflower seed oil and in sunflower seed fat obtained by hydrogenation under selective and inselective conditions.

The triglycerides were separated on silver nitrate impregnated silica gel layer. The fatty acid composition of the separated fractions was determined by gas chromatography. The percentage of the weight of individual triglyceride fractions in the oil was quantitatively determined by adding known quantities of pure arachidic acid to the sample.

The triglyceride groups containing fatty acids of different degrees of saturation were calculated from the fatty acid composition of the fractions.

By means of this method nine triglyceride groups were detected in natural sunflower seed oil.

Investigation of the triglyceride groups in the products of roughly identical slip points (29 °C), obtained by selective and inselective hardening of sunflower seed oil, has led to the following conclusions:

— about 85% of the sunflower seed fat obtained by selective hardening is made up of triglyceride molecules whose three fatty acid components contain altogether 3 or 4 double bonds (*e.g.* monolinol-diolein, triolein);
— inselectively hardened sunflower seed oil contains about 60% of triglyceride molecules in which the three fatty acid components contain altogether 3 or 4 double bonds;
— in the product of inselective hardening more triglyceride groups of various degrees of saturation are found in a relatively more even distribution than in the end-product of selective hardening.

The physical and chemical parameters of the various vegetable and animal oils and fats are determined by the degree of saturation and chain length of their fatty acids and the positions of the latter in the triglyceride molecule. When vegetable oils are hydrogenated the fatty acid components are partly or totally saturated and isomerized, consequently the triglyceride structure and the parameters are modified.

Description of the triglyceride structure requires complex analytical testing, since the various fats contain a great variety of triglyceride molecules whose properties, including their molecular weights, differ only slightly. Thus, *e.g.* five fatty acid components may form 35 triglyceride molecules of different fatty acid composition, or, if a distinction is made between the terminal and middle position of glycerin, 75 triglyceride molecules of different structures.

The investigation of the glyceride structure of natural sunflower seed and linseed oil dates back in our Institute to the beginning of the 1950's (JÁKY & PERÉDI, 1951). The geometric and positional isomers formed in the side-reactions of hydrogenation will greatly raise the number of triglyceride components. This is presumably the reason for the lack of a detailed study and the description of the triglyceride structure of hydrogenated fats in the literature. Several methods have been worked out for the study of the triglyceride components of natural fats, of which the diverse fractionation methods, oxidation and pancreatic lipase hydrolysis are the most important. The fractionation methods include counter-current distribution (EVANS *et al.*, 1966), thin-layer chromatography (KAUFMANN *et al.*, 1961; DE VRIES & JURRIENS, 1964), column chromatography (DE VRIES, 1963) and gas chromatography (BEZARD *et al.*, 1971). These methods enable the separation of fractions according to their degree of unsaturation and to the number of their carbon atoms. The triglyceride structure can be described from the ratio of the fatty acid components of the various fractions. With the oxidation methods conclusions are drawn from the products as regards the structure of the triglyceride components (YOUNGS & SUBBARAM, 1964; KARTHA, 1969). By means of pancreatic lipase hydrolysis it is possible to separate the monoglyceride fractions in beta position and thereby the fatty acid components in the middle position of the glycerides (VANDER WAL, 1960). By VANDER WAL's (1960) method of calculation, presuming a 1,3-random-2-random distribution, the triglyceride components can be calculated from the overall fatty acid composition of the sample under investigation and from that of the beta-monoglycerides (VANDER WAL, 1963).

These methods may also be applied to hardened products when complemented with analytical procedures — such as *e.g.* open tubular gas chromatography (LITCHFIELD & REISER, 1963; SCHOLFIELD *et al.*, 1967) suitable for the determination of the geometric and positional isomers of fatty acids formed during hydrogenation.

The first objective of our investigations was the determination of triglyceride groups differing in their degree of saturation by thin-layer chromatography. DE VRIES and JURRIENS (1964) worked with silica gel impregnated with silver nitrate as thin layer on which the triglyceride molecules could be separated according to the number of unsaturated bonds in their structures. Separation is brought about by the π-complexes formed between the unsaturated bonds and the silver ions (MORRIS, 1966). WESSELS and RAJAGOPAL (1969) found later that the retention distance depends not alone on the number of double bonds, but also on their position in the molecule and on their geometric configuration. For instance, the retention distance of the triolein molecule containing three double bonds is greater than that of the monolinolene-distearin molecule which also contains 3 double bonds, on the other hand

the retention distance of trielaidin which has a *trans*-configuration, is somewhat greater than that of triolein containing double bonds in *cis*-configuration.

With the help of this method it is possible to establish the characteristic triglyceride molecules of different degrees of saturation in the substance under investigation. The method, however, fails to provide an answer to the question of the position of fatty acids in the marginal and middle positions of the various molecules, further on the distribution of palmitic and stearic acid between the molecules.

In this work we studied the change in the triglyceride groups of various degrees of saturation and in their distribution during the hydrogenation of sunflower seed oil. The changes in the triglyceride structure of sunflower seed oil as a result of hardening were investigated in the case of two plant-scale hydrogenations. In one case the conditions of hydrogenation favoured selective (samples marked I), in the other inselective (samples marked II) hardening. With respect to selectivity we have accepted ALLEN's (1960) interpretation according to which selectivity should mean chemical selectivity, that is, the step-by-step saturation of the unsaturated fatty acid components of the triglyceride molecules.

1. Materials and methods

1.1. Materials

Natural sunflower seed oil and sunflower seed fat obtained by hydrogenation under different conditions were investigated. The hardened samples came from a *Normann*-system plant intermittently operating. A copper-nickel catalyst with 20 to 25% of Ni content was used.

Selective hardening of the sunflower seed oil was performed at temperatures between 180 and 200 °C with a moderately active catalyst (50% of fresh catalyst and 50% of a catalyst used already once in a hardening process), under over-pressure and without agitation (samples I). The total period of hydrogenation was 4 hours, resulting in a sunflower seed fat with a slip point of 29.1 °C (sample I/4) as the end-product. Beside the latter two intermediary samples, one taken in the 90th minute (sample I/2) and the other in the 180th minute (sample I/3) of hardening were also tested.

The inselective hardening of sunflower seed oil (samples II) was performed at temperatures between 110 and 160 °C with fresh active catalyst at 0.7 gauge pressure. The investigated end-product produced in 165 minutes had a slip point of 28.9 °C, but two intermediary products were also tested; one was a sample taken in the 95th minute of hardening (sample II/2), the other in the 135th minute (sample II/3).

1.2. Test methods

1.2.1. Thin-layer chromatography for the purification of triglycerides. The triglyceride samples to be investigated were freed from the diverse decomposition products, including the non-saponifiable components and free fatty acids, prior to fractionation. Purification was performed on a Kieselgel G layer with petrol ether — ether — acetic acid of 90 : 10 : 1 (v/v) as a lipid-group-separating solvent system according to MALINS and MANGOLD (1960). The oil samples were applied as narrow stripes to the layer. After separation the components were made visible in the marginal band of the layer by means of iodine vapour. The zone containing the triglycerides was scraped off the plate and eluted from the silica gel with ether.

1.2.2. Separation of triglyceride groups by means of thin-layer chromatographic analysis. Separation of the triglyceride groups was performed on the Kieselgel G layer impregnated with 10% (w/w) of silver nitrate with a solvent system consisting of chloroform and methanol in the proportion of 98 : 2, or 99 : 1 (v/v). 25 to 30 mg of purified triglyceride was applied in the form of a narrow stripe to each plate. At the end of the run the fractions were made visible in the UV light by treatment with dichlorofluorescein. The zones containing the diverse triglyceride groups were scraped off the plates.

1.2.3. Preparation of the methyl esters. The fatty acid components of the triglycerides adsorbed on the silica gel were converted into methyl esters by boiling with a solvent mixture consisting of methanol, benzene and sulphuric acid, 75 : 25 : 4 (v/v), for 2 hours. The methyl esters were then extracted from the esterifying mixture with petrol ether – ether, 1 : 1 (v/v). Prior to esterification exactly 1 mg of gas chromatographically pure methyl arachidate was added to each triglyceride fraction (BLANK *et al.*, 1965).

1.2.4. Gas chromatography of the methyl esters. The gas chromatographic analysis of the methyl esters was performed in a PYE UNICAM apparatus, under the following conditions:

Length of column: 1.8 m
Internal diameter of column: 0.4 cm
Support: Chromosorb W (100 to 120 mesh)
Stationary phase: 10% of ethyleneglycol succinate
Temperature: of the thermostat 188 °C, of the detector 240 °C, of the evaporator 240 °C
Detector: flame ionization
Carrier gas: nitrogen, 50 ml min^{-1}.

In addition to the fatty acid composition, the chromatograms could be used to determine the quantities of the fractions from the area under the peak of the known quantity of methyl arachidate.

1.2.5. Determination of isolated trans-isomer fatty acid content. The *trans*-isomer fatty acid content was determined according to the IUPAC method published in 1970 with the IR spectrophotometer ZEISS UR 20.

2. Results

2.1. Natural sunflower seed oil

The pure sunflower seed oil used as the base material of hydrogenation gave nine fractions on the silver nitrate impregnated layer. The proportion of the fractions and the distribution of the fatty acid components among the fractions are shown in Table 1.

The table contains the fractions in the order as they appear on the layer. No.1 is the lowest fraction with the smallest retention distance, No.9, the uppermost fraction, has the greatest retention distance.

The types of triglycerides in the various fractions were determined from the fatty acid composition of the fractions with the help of literature data (WESSELS & RAJAGOPAL, 1969; KAUFMANN & WESSELS, 1964). Distribution according to percentage (w/w) of triglycerides within the fractions was determined from the fatty acid compositions using a system of equations of the first order with several unknowns. The distribution of the triglyceride groups

Table 1

Fatty acid composition of the triglyceride components of sunflower seed oil after separation on thin-layer and the percentage (w/w) distribution of the fractions

[Layer: Kieselgel G impregnated with 10% (w/w) of $AgNO_3$,
Solvent: chloroform—methanol, 98 : 2 (v/v)]

Name	Fatty acid components				Triglyceride fraction, %
	Palmitic acid, %	Stearic acid, %	Oleic acid, %	Linoleic acid, %	
Original oil	5.7	3.9	25.2	65.2	—
Fraction 1	—	—	2.5	97.5	26.6
Fraction 2	1.2	0.3	32.2	66.3	27.4
Fraction 3	15.3	12.8	4.9	67.0	15.8
Fraction 4	2.1	1.6	62.9	33.4	11.7
Fraction 5	17.2	10.5	36.0	36.3	10.1
Fraction 6	2.8	2.0	85.6	9.6	2.6
Fraction 7	29.1	18.4	22.2	30.3	2.4
Fraction 8	16.3	14.9	60.2	8.6	2.7
Fraction 9	30.2	22.9	46.9	—	0.7

in the original oil was obtained from the above data and from the ratio of fractions.

Using this method the maximum standard deviation between the results of five parallel analyses is $s_{\bar{x}} = \pm 0.69$. The probable value of the quantity of single triglycerides (M) is, from the average ($\bar{X}$) of three parallel determinations, in case of P = 95%, $M = \bar{X} \pm 1.13$. In these calculations no distinction was made between palmitic acid and stearic acid, in other words, the total quantity of the two was considered as the total saturated fatty acid content.

The distribution of the fatty acid components in the fractions indicates their main triglyceride component. For instance, in the first fraction trilinol (LLL), in the second dilinol-monoolein (LLO) is the main component. In each fraction small quantities of the triglyceride components characteristic of the two adjacent fractions are also present. In the third fraction dilinol-monosaturated (LLS) is the component occurring in the highest quantity, in the fourth fraction mono-linol-diolein (LOO). Separation of these two triglyceride groups shows clearly that of the two molecules with identical numbers of double bonds (both having altogether four unsaturated bonds) the one with the fatty acid component containing more double bonds will have the smaller retention distance.

The percentage distribution of the triglyceride groups in sunflower seed oil is shown in Table 2.

Table 2

Distribution of the various triglyceride groups in sunflower seed oil, % (w/w)

(S = saturated fatty acids, O = oleic acid, L = linoleic acid)

Type of triglyceride	% (w/w)
OSS	0.4
OOS	1.4
LSS	1.9
OOO	3.0
LOS	11.0
LOO	12.5
LLS	14.6
LLO	30.4
LLL	24.8

The results are almost identical with the results to be found in the literature on sunflower seed oil (GUNSTONE & QUERESHI, 1965).

2.2. *Hydrogenated sunflower seed oil*

The hydrogenated samples were subjected to the same tests as the original sunflower seed oil, while the polarity of the fractionating solvent system had to be changed because of reduced unsaturation of the samples. The fractions did not separate quite as sharply on the thin-layer as in the case of natural sunflower seed oil, due probably to the slight difference between the retention distances of triglycerides containing *trans*-isomer fatty acids formed during the hydrogenation and the retention distances of the triglycerides containing *cis*-fatty acids of the same carbon number and of the same degree of saturation.

The fatty acid composition, *trans*-isomer fatty acid content and slip point of the end-product (sample I/4) produced by the hardening of sunflower seed oil by process I, as well as of the two intermediary products (samples I/2 and I/3) of hardening and of the sunflower seed oil used as base material (sample I/1) are shown in Table 3.

Table 3

Changes in the fatty acid composition and slip point of sunflower seed oil during hardening of type I (of selective character)

Conditions of hardening: temperature: 180 to 200 °C,
oil: 7 500 kg
hydrogen: 140 m^3h^{-1}
catalyst: 75 kg of fresh and 75 kg of once used Ni-Cu catalyst with 20 to 25% Ni content

Fatty acid component	Samples			
	I/1	I/2	I/3	I/4
Palmitic acid	5.7	5.9	6.0	6.1
Stearic acid	3.9	4.9	6.3	6.8
Oleic acid	25.2	35.3	58.1	72.3
Linoleic acid	65.2	53.9	29.6	14.8
Trans fatty acid	—	14.8	50.0	58.3
Slip point, °C	—	—	25.2	29.1

The distribution of the triglyceride components determined from the fractions separated on the thin-layers are presented in Table 4 and Fig. 1.

Table 5 contains the data on the fatty acid composition, *trans*-isomer fatty acid content and slip point of the end-product (sample II/4) obtained by process II of hardening of the sunflower seed oil, as well as those of the two intermediary products (samples II/2 and II/3) and of the sunflower seed oil used as base material (sample II/1).

Table 4

Percentage (w/w) distribution of triglyceride groups with different degrees of saturation of their fatty acid components in samples taken from the selective (type I) hardening of sunflower seed oil

(S = saturated fatty acids, O = oleic acid, L = linoleic acid)

Type of triglyceride	Percentage (w/w) distribution of triglyceride groups			
	Number of samples and time of sampling			
	I/1 (0 min)	I/2 (90 min)	I/3 (180 min)	I/4 (240 min)
SSS	—	—	0.3	0.4
OSS	0.4	0.5	3.3	3.8
OOS	1.4	6.9	9.9	10.0
LSS	1.9	2.8	3.7	3.9
OOO	3.0	8.6	22.3	40.1
LOS	11.0	13.8	16.8	11.0
LOO	12.5	18.3	22.4	28.6
LLS	14.6	11.6	3.6	0.5
LLO	30.4	23.3	16.1	1.7
LLL	24.8	14.2	1.6	—

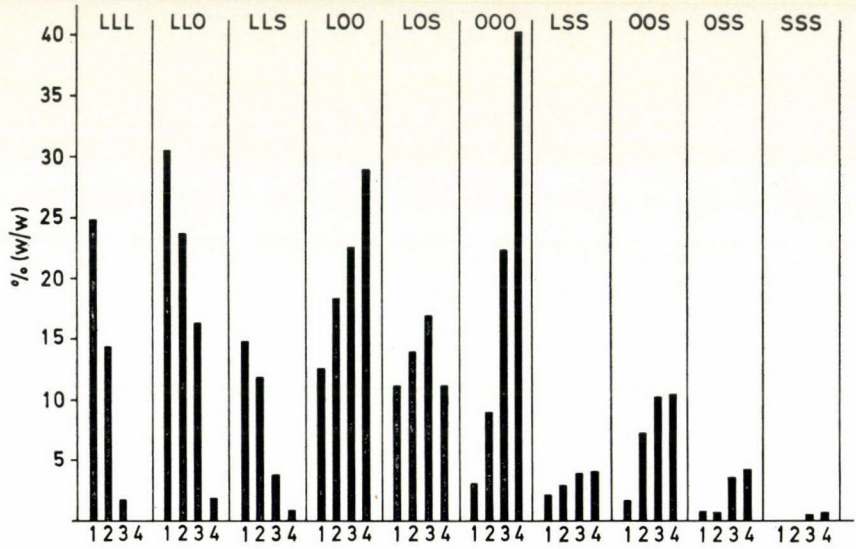

Fig. 1. Changes in the triglyceride groups of sunflower seed oil during hardening of type I (selective hardening). (Based on data from Table 4). Sample 1: natural sunflower seed oil, the base material of hardening. Sample 2: taken in the 90th minute of hardening. Sample 3: taken in the 180th minute of hardening. Sample 4: taken in the 240th minute of hydrogenation, the end-product of hardening. (S = saturated fatty acids, O = oleic acid, L = linoleic acid)

Table 5

Changes in the fatty acid composition and slip point of sunflower seed oil during inselective (type II) hardening

Conditions of hardening: temperature: 110 to 160 °C
oil: 7 500 kg
hydrogen: 140 $m^3\ h^{-1}$
catalyst: 100 kg fresh Ni-Cu catalyst with 20 to 25% Ni content

Fatty acid components	Samples			
	II/1	II/2	II/3	II/4
Palmitic acid	5.7	6.2	6.5	6.8
Stearic acid	3.9	5.4	9.7	10.5
Oleic acid	25.2	31.9	47.8	60.2
Linoleic acid	65.2	56.5	36.0	22.5
Trans-fatty acid	—	9.8	20.8	35.6
Slip point, °C	—	—	23.2	28.9

Table 6 and Fig. 2. illustrate the distribution of the triglyceride components determined from the fractions separated on thin-layers.

Table 6

Percentage (w/w) distribution of triglyceride groups with different degrees of saturation of their fatty acid components in samples taken from the inselective (type II) hardening of sunflower seed oil

(S = saturated fatty acids, O = oleic acid, L = linoleic acid)

Type of triglyceride	Percentage (w/w) distribution of triglyceride groups			
	Number of samples and time of sampling			
	II/1 (0 min)	II/2 (75 min)	II/3 (135 min)	II/4 (165 min)
SSS	—	—	1.0	1.3
OSS	0.4	2.3	3.9	4.9
OOS	1.4	5.7	9.2	16.6
LSS	1.9	2.2	3.9	2.6
OOO	3.0	7.3	21.6	26.6
LOS	11.0	9.6	11.6	12.2
LOO	12.5	12.5	18.2	20.4
LLS	14.6	12.8	8.2	3.8
LLO	30.4	28.3	22.0	10.6
LLL	24.8	19.3	0.4	—

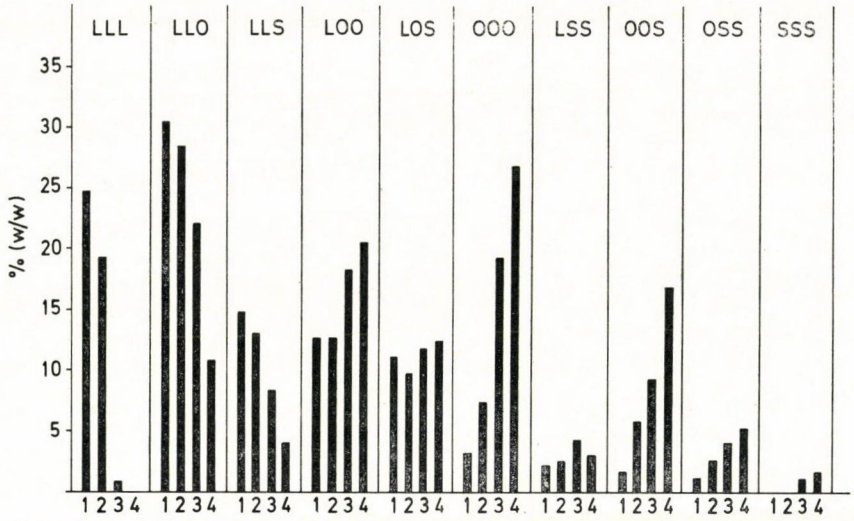

Fig. 2. Changes in the triglyceride groups of sunflower seed oil during hardening of type II (inselective hardening). (Based on data from Table 6). Sample 1: natural sunflower seed oil, the base material of hardening. Sample 2: taken in the 75th minute of hardening. Sample 3: taken in the 135th minute of hardening. Sample 4: taken in the 165th minute of hydrogenation, the end-product of hardening. (S = saturated fatty acids, O = oleic acid, L = linoleic acid)

3. Conclusions

Under the conditions of hydrogenation I, favouring selective saturation of the triglyceride molecules (Fig. 1), the quantity of the most unsaturated components of sunflower-seed oil, namely that of trilinol (LLL) and dilinol-monoolein (LLO) gradually decreased, while the quantities of the monolinol-diolein (LOO) and of the triolein (OOO) fractions increased. The dilinol-mono-saturated (LLS) molecules became gradually saturated, while the diene components were converted into monoenes. The end-product of hardening contained mainly triglyceride molecules whose three fatty acid components contained altogether 3 or 4 double bonds. These fatty acid components were monolinol-diolein (LOO), monolinol-monoolein-mono-saturated (LOS), and triolein (OOO), and their contribution amounted to about 85% of the hardened oil. At the same time the overall quantity of trilinol (LLL) and of dilinol-monoolein (LLO), considered the main components of sunflower seed oil, dropped from the initial 55% below 2%.

Under the conditions of hydrogenation of type II the quantity of the most unsaturated components of the sunflower-seed oil decreased gradually (Fig. 2), moreover the trilinol (LLL) molecules disappeared completely, similarly to the selective type hydrogenation I, while the quantity of triglyceride

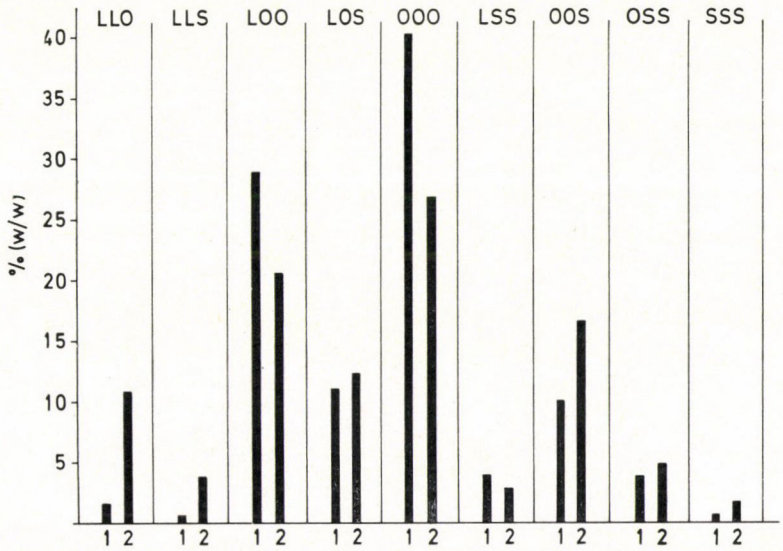

Fig. 3. Distribution of the triglyceride groups of sunflower seed oil after hardening by process I (selective type of hardening) and by process II (inselective type of hardening), resp. Sample 1: end-product of hardening by process I. Sample 2: end-product of hardening by process II. (S = saturated fatty acids, O = oleic acid, L = linoleic acid)

molecules containing altogether 3 or 4 double bonds — such as of monolinoldiolein (LOO) and of triolein (OOO) — increased. The latter two components amounted to about 60% of the end-product.

From Fig. 3, in which the triglyceride components of the end-products of practically identical slip points (29 °C) obtained by hydrogenation under two different sets of conditions are shown, the following conclusions can be drawn: Under the conditions of hydrogenation of type II the sunflower seed oil is saturated in a more inselective manner than under the conditions of hydrogenation of type I. This is indicated by the higher stearic acid and higher linoleic acid content of end-product II (Tables 3 and 5), further by the occurrence of 6 different triglyceride groups in quantities above 4% and in a relatively more even distribution (between 4 and 27%), while in the end-product of the hydrogenation of type I only 4 such triglyceride groups are present (in quantities between 4 and 40%).

However, the fact that trilinol molecule was not found in the end-product of hardening of type II either, and that triolein occurred in the highest quantity, indicates that under the given conditions, beside inselective saturation, some saturation of the selective type has also taken place.

*

The authors wish to express their thanks to Mr. J. JUHÁSZ, plant engineer of the *Rákospalotai Növényolajgyár*, for his courtesy in permitting to take samples from the plant.

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Acta Alimentaria, Vol. 4. (2) pp. 151—165 (1975)

EXPERIMENTS TO INCREASE THE JUICE YIELD OF GRAPES BY RADIATION TREATMENT

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(Received December 27, 1973)

The effect of irradiation at different dose levels (50, 200, 800 and 1 600 krad) on the juice yield and on the composition and organoleptic quality of the wine, fermented from the juice, was studied in seven experimental series. All experiments were carried out in 3 replicates.

The juice yield of the treated grapes increased with increasing dose level. The maximum increase in yield was 28% in comparison to that of the control. On the average a linear correlation was found between the square root of dose and the percentage increase in yield.

At higher dose levels a change in consistency, darkening of the colour, softening and thinning of the fruit skin and stem and the liquefaction of the fruit was observed. Fermentation was neither retarded nor slowed down by radiation treatment.

The composition of wines gained from the fermented juice of treated grapes, particularly those treated with high doses, differed to a certain degree from that gained from untreated fruit. Increased extract, ash content, pH, index of polyphenols and colour intensity were observed. The change was particularly significant and unfavourable in wines gained from grapes treated with 800 and 1 600 krad, respectively. These had an unpleasant odour and flavour of oxidized, foreign character.

Thus, treatment with 800 and 1 600 krad, proved undesirable. Further experiments are required to test the effect of lower doses between 200 and 500 krad.

The application in the food industries of radiation treatment for various purposes is gradually gaining ground. The prime aim of irradiating fruits is to prolong their storage life, to preserve them. However, some side-effects, such as the increase of juice yield upon processing, are not negligible either. This effect is particularly important as regards grapes. The increase of grape juice yield desirably reduces prime cost, improves economic efficiency. However, the increase in yield must not impair the quality of must or wine. The juice yield may be increased by the application of presses of new construction or of pectolytic enzymes, but radiation treatment is also a possibility.

The increase of juice yield depends on the compressibility of the grape, which in turn depends on the quantity and quality of interlamellar pectin content of the grapes. As an effect of irradiation pectin breaks down easily and at the same time the tissues are also damaged and thereby their compressibility is improved and the juice yield increased.

Relatively few data may be found in the literature on the juice yield of grapes as affected by ionizing radiations. Soviet authors (KOVAL'SKAYA *et al.*,

1961; VASILEVA *et al.*, 1963) found a juice increase of 10% upon treatment with 400—600 krad. This was considered to be due to the impairment of the skin structure, of the correlation between skin and flesh and to the improved permeability of the tissues. ROGACHEV (1966) found that the shelf-life of certain fruits could be extended by radiation treatment. Upon treatment with 300 krad the storage life of grapes was extended by 10—15 days at 25 °C. When irradiated with doses above 400 krad yellow or brown discolouration and a reduced immunity to subsequent infection was observed. As an effect of treatment the yield of grapes increased by 7—10%.

FRUMKIN and NAKHMEDOV (1970) observed an increase of 7—16% in juice yield upon treatment with 100—500 krad and the quality of dry table wines was also improved. The juice yield of apple was increased by 3—8% upon irradiation with 50—300 krad.

MASSEY (1968) investigated the mechanism of irradiation effect and found that first the semipermeable membran of the cells was damaged and the degradation of pectin and cellulose was also promoted.

MALYSHEV and ROGACHEV (1970) found that irradiation played an important role in the mechanism of degradation of the cell walls, both during the period following treatment and during storage as well.

During 1970 preliminary experiments were carried out to increase the juice yield of grapes by radiation treatment and an increase of 12—15% was achieved. On the basis of these results our Institute was commissioned by the *Central Food Research Institute* to investigate the effects of radiation treatment upon juice yield, upon the process of fermentation, upon the composition of wines gained from the juice of radiation treated grapes and upon their organoleptic quality.

1. Materials and methods

1.1. Place of cultivation and variety of grapes

The equalized batches of grapes were obtained from the Research Stations of the *Research Institute for Viticulture and Enology* at Miklóstelep and Eger. The radiation treatment was carried out in the irradiation plant of the *Central Food Research Institute* and the experiments in the laboratories of the *Research Institute for Viticulture and Enology.*

The grape varieties tested were as follows:

Eger	*Egri csillagok*
	Olasz rizling
Miklóstelep	*Kocsis Irma*
	Kövidinka
	Hárslevelű (on 3 occasions)

1.2. Conditions of irradiation

The freshly picked grapes were treated within 3—5 hours in the Experimental Irradiation Plant of the *Central Food Research Institute* (Budapest). The nominal activity of the ^{60}Co radiation source is 60 kCi and the dose rate 180 krad h^{-1}.

The radiation doses applied were: 0, 50, 200, 800 and 1 600 krad, respectively.

1.3. Experimental conditions

Of each variety 100 kg of fruit at the same stage of ripeness were picked and sent immediately to the irradiation plant. 20 kg of grapes were irradiated at each dose level. The irradiated samples were then individually pressed. Five-kg lots in three replicates were pressed at each treatment level and of the untreated control sample.

The samples were marked as follows:

for the place of origin		for variety	
M	Miklóstelep	*K*	*Kocsis Irma*
E	Eger	*Kö*	*Kövidinka*
		H	*Hárslevelű*
		E	*Egri csillagok*
		O	*Olasz rizling*

Index numbers *1*, *2* and *3* adjoining *H* (Hárslevelű) stand for first, second and third harvest of the grape variety at about 2-week intervals.

1.4. Methods for testing radiation effect

1.4.1. Pressing method. Five-kg lots of grapes were stalked and pressed under stable conditions at about 3—4 kp cm^{-2} pressure for 45 minutes, pulling the screw about every five minutes.

1.4.2. Testing of the samples. The exact weight of stems, of the berries and of the marc, the volume of juice, the specific weight of must were established. The weight and percentage of juice yield (juice, litre/grapes, g) were calculated. The increase in juice yield relative to the control was established. The average deviation amounted to about 4.5%.

1.4.3. Observation of the fermentation process. The must of the 3 parallel samples was equalized and fermented. The effect of radiation treatment upon the commencement of fermentation and the process itself were observed to see whether it was retarded or inhibited by irradiation.

1.4.4. Chemical and organoleptic investigation of the wines obtained. Every experimental sample was evaluated by a panel of judges and the following characteristics were tested: alcohol; total extract; invert sugar; extract, free of sugar; ash; alkalinity of the ash; titratable acid content; pH; the free and total SO_2 content; colour intensity and polyphenol content.

These tests were carried out to see the changes relative to the control in the wines obtained from radiation treated grapes.

The wine samples were tested also for filterability.

2. Results

2.1. The condition of grapes after irradiation

The grapes treated with 50 krad did not differ from the control. Similar observations were made with grapes treated at the 200 krad level. However, two of the samples (MH_2 and MH_3) showed some brown discolouration and the fruit became slightly fragile (Tables 6 and 7).

Out of the samples treated with 800 krad three showed no signs of change, in four cases, however, a brownish discolouration of the fruit, softening and thinning of the stems and skins was observed (MH_1, MH_2, MH_3 and EO; Tables 2, 5, 6 and 7).

Every grape variety suffered significant changes upon treatment with 1 600 krad. The fruit became definitely brown, the stems and skins became soft and thin and fragile. The tissue broke down and the grapes became liquified, very difficult to stalk. In three cases (MH_1, MH_2 and MH_3) juice was observed at the bottom of the crates (Tables 5, 6 and 7).

Table 1

Juice yield of variety "Egri csillagok", Eger (EE) upon irradiation at various dose levels (1971)

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	161	4 838	1 640	2 807	56.1	0
50	176	4 823	1 470	3 007	60.1	7.1
200	171	4 828	1 436	3 060	61.2	9.1
800	163	4 837	1 163	3 270	65.4	16.5
1 600	153	4 847	1 081	3 396	67.9	20.9

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of the 3 parallel samples.

* litres per 100 kg

Table 2

Juice of grape variety "Olasz rizling", Eger (EO) upon irradiation at various dose levels (1971)

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	284	4 716	1 235	3 050	61.9	0
50	301	4 698	1 215	3 156	63.1	2.1
200	253	4 747	1 172	3 243	64.9	5.0
800	306	4 693	1 198	3 210	64.2	4.0
1 600	326	4 683	1 150	3 226	64.3	4.4

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg.

Table 3

Juice yield of grape variety "Kocsis Irma", Miklóstelep (MK) upon irradiation at various dose levels (1971)

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	180	4 813	1 860	2 603	52.0	0
50	168	4 831	1 836	2 706	54.1	4.0
200	181	4 818	1 673	2 800	56.0	7.6
800	175	4 825	1 490	2 990	59.8	14.9
1 600	180	4 820	1 202	3 253	65.1	25.0

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg.

Thus the various grape varieties reacted differently to radiation treatment. The most resistant varieties were: *Kocsis Irma*, *Kövidinka* and *Egri csillagok*. These were affected only at the highest dose level. Slightly less resistant was *Olasz rizling*. *Hárslevelű* proved to be the most sensitive variety, particularly at a more advanced stage of ripeness. The latter was affected even by treatment with 200 krad.

The colour changes are shown on colour-plates made from the treated and the control samples.

Table 4

Juice yield of grape variety "Kövidinka", Miklóstelep (MKö) upon irradiation at various dose levels (1971)

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	200	4 800	1 785	2 703	54.1	0
50	211	4 788	1 745	2 770	55.4	2.3
200	231	4 768	1 436	3 013	60.3	11.5
800	228	4 772	1 220	3 213	64.3	18.8
1 600	196	4 803	988	3 476	69.5	28.6

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg.

Table 5

Juice yield of grape variety "Hárslevelű", Miklóstelep (MH_1), first harvest (12. 9. 1971), upon irradiation at various dose levels

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	247	4 753	1 590	2 830	56.6	0
50	255	4 745	1 675	2 790	55.8	0
200	253	4 747	1 543	2 883	57.7	1.9
800	335	4 665	1 108	3 207	64.1	13.3
1 600	315	4 685	1 005	3 355	67.1	18.5

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg.

Figs. 1, 2 and 3 show the fruit of the *Hárslevelű* variety from Miklóstelep from the third harvest.

On the basis of the above observations the wines obtained from fruit treated at high dose levels were expected to differ in their composition, treatability and organoleptic qualities from those gained from untreated fruit.

Table 6

Juice yield of grape variety "Hárslevelű", Miklóstelep (MH_2), second harvest (24. 9. 1971), upon treatment at various dose levels

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	210	4 790	1 663	2 763	55.3	0
50	201	4 799	1 639	2 835	56.7	2.6
200	220	4 780	1 395	3 046	60.9	10.2
800	226	4 773	1 262	3 130	62.6	13.3
1 600	254	4 763	892	3 466	69.3	25.8

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg .

Table 7

Juice yield of grape variety "Hárslevelű", Miklóstelep (MH_3), third harvest (6. 10. 1971), upon irradiation at various dose levels

Radiation dose (krad)	Weight of stems (g)	Weight of grapes (g)	Weight of marc (g)	Juice yield (ml)	Juice yield % (v/w)*	Increase of juice yield (%)
0	215	4 785	1 776	2 610	52.2	0
50	223	4 777	1 778	2 663	53.3	2.0
200	215	4 785	1 636	2 763	55.3	5.9
800	281	4 718	1 198	3 153	63.1	20.8
1 600	226	4 773	1 066	3 343	66.9	28.1

Experimental batches of 5 000 g were used throughout. Data given in the Table are averages of 3 parallel tests.

* litres per 100 kg.

2.2. Juice yield

The seven grape varieties were treated at four dose levels in three replicates, each. The experimental data related to the controls are given in Tables 1—7.

Theoretically the weight of marc and juice should correspond to the original weight of the stalked fruit. In reality losses arising during operations cause in the case of a small press, a relatively large difference in the weights. In a 5-kg batch the difference amounted to about 150 g, on the average.

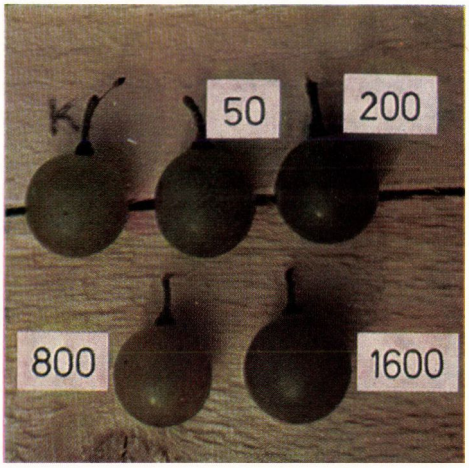

Fig. 1. *Hárslevelű* grape variety from Miklóstelep. Third harvest, treated(150 — 1 600 krad) and control samples

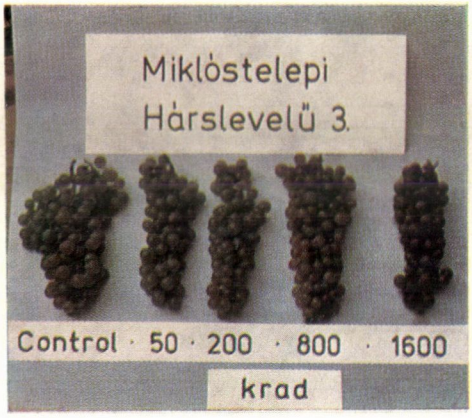

Fig. 2. *Hárslevelű* grape variety, Miklóstelep. Third harvest, treated and untreated

Fig. 3. *Hárslevelű* grape variety, Miklóstelep. Third harvest, treated and untreated

As seen from the results, the juice yield of grapes increased upon irradiation and the increase was in direct proportion to dose level. When treated with 1 600 krad, all the samples, except *EO* (Table 2), gave a yield increase of above 18%. Treated with 800 krad, the samples, except *EO*, showed 13% increase in juice yield. The increase in juice yield and the averages, for all the samples, are given in Table 8.

Table 8

Percentage increase in juice yield of various radiation treated grape varieties, related to the untreated sample

Radiation dose (krad)	*Egri csillagok*, Eger (*EE*)	*Olasz rizling*, Eger (*EO*)	*Kocsis Irma*, Miklóstelep (*MK*)	*Kövidinka*, Miklóstelep (*MKö*)	*Hárslevelű 1*, Miklóstelep (MH_1)	*Hárslevelű 2*, Miklóstelep (MH_2)	*Hárslevelű 3*, Miklóstelep (MH_3)	Average of the 7 grape varieties
50	7.1	2.1	4.0	2.3	0	2.6	2.5	2.9
200	9.1	5.0	7.6	11.5	1.9	10.2	5.9	7.3
800	16.5	4.0	14.9	18.8	13.3	13.3	20.8	14.5
1 600	20.9	4.4	25.0	28.6	18.5	25.8	28.1	21.6

Data given in the Table represent average values of 3 parallel tests, of 5 000 g original weight each.

Treatment with 50 krad resulted in a relatively small increase of juice yield, about 3%. Upon treatment with 200 krad the yield increased by about 7%, while upon treatment with 800 krad by 14% and at 1 600 krad by 21%.

It should be noted that the average juice yield, due to the method of pressing, was rather low in these experiments. However, results are not affected by this fact, because pressing was carried out under exactly the same conditions every time.

The correlation between juice yield and treatment, as the average for each variety, is graphically illustrated in Fig. 4. The dose level is shown on the abscissa in krad, and the percentage increase in juice yield on the ordinate.

The increase in juice yield is not linear to the dose level.

Fig. 5 shows the square root of the radiation dose on the abscissa.

As seen in the figure, the correlation between juice yield and the square root of radiation dose is linear. The equation of the first order, expressing the correlation, is as follows:

$$Y = 0.5\sqrt{\mathrm{d}},$$

where

Y = increase of juice yield, %,

$\sqrt{d}$ = square root of the dose in krad.

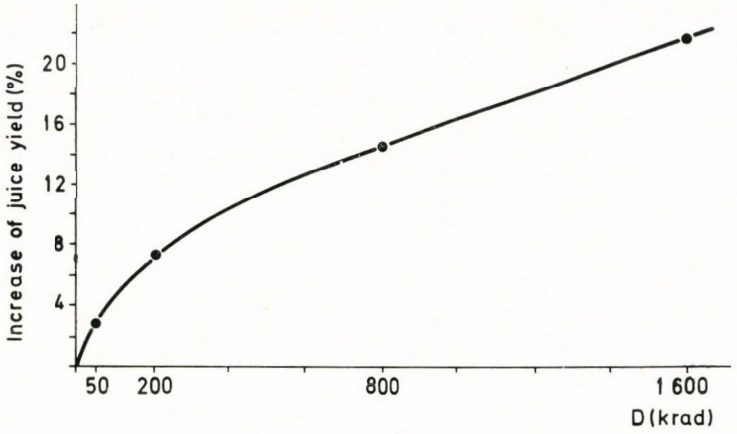

Fig. 4. Increase in juice yield as affected by dose level

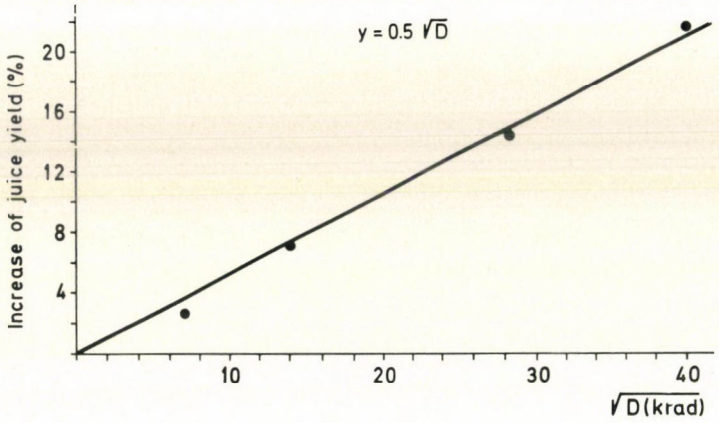

Fig. 5. Correlation between the square root of radiation dose and the increase in juice yield

2.3. The fermentation process

The must gained from each variant was equalized and fermented at room temperature. The samples received the usual amount of SO_2 (50 – 100 mg l^{-1}) but were not inoculated with a specific yeast variety. The process of fermentation was normal in almost every case, there was no substantial difference observable between the control and the samples undergone treatment. Only the juices of samples *MK* treated with 1 600 krad and of MH_1 treated with 800 krad were not completely fermented. The residual sugar in the former amounted to 41 g l^{-1}, in the latter to 7 g l^{-1}. The control sample of MH_1 variety did not undergo fermentation at all. The reason for this phenomenon is not known.

In all the other samples fermentation started normally and came to an end in 5 to 7 days. No unfermented sugar remained.

The observations permit of the conclusion that the radiation doses applied did not destroy the natural yeast population of the grapes, did not retard or inhibit fermentation. The residual yeast population was sufficient to start and complete fermentation in the majority of samples.

2.4. *Chemical composition and organoleptic quality of the wines obtained from the treated and control samples*

The results of the chemical analysis of wines obtained in the experiments are summarized in Table 9.

Though substantial difference was not found in the composition of wines obtained from treated fruit, a slight increase in the sugar-free extract and ash content of these wines was observed. In general the pH value increased with increasing radiation dose levels.

Data of the polyphenol-index and colour intensity were of extreme interest. The polyphenol-index was calculated from the absorption value (optical density (A_{280})) as measured in a twenty-fold dilution at 280 nm. The colour intensity was established directly in the wine, measured at 420 nm wavelength in a cuvette of 1 cm width (A_{420}). The polyphenol-index, as well as the colour intensity increased with increasing radiation dose. The colour tended toward an oxidized character.

The wines obtained from grapes treated at the highest dose level reminded to a certain extent of wines fermented from must of additional pressing.

All the wine samples were subjected to organoleptic test by an expert panel. It was firmly established that wines gained from grapes treated with 800 krad or in particular with 1 600 krad were of a foreign character, of oxidized taste and odour, and of mousiness. With some of the varieties the change became more apparent in the taste, in others in the odour. The wines from grapes treated with 200 krad varied. Some of them suffered no unfavourable change, while others showed the same characteristics as those treated at higher levels, only to a lesser degree. Samples given a 50-krad treatment were not different from the control.

The tannic acid—gelatine method was used to clarify the wines after the end of fermentation. Then they were tested for filterability. However, these test did not permit of drawing conclusions. With some of the varieties the untreated sample was most suitable for filtration, with others these were the least suitable. Thus related data are not published.

Table 9

Chemical analysis of wines obtained

Location and grape variety	Radiation dose (krad)	Alcohol (% v/v)	Total extract (g l^{-1})	Invert sugar (g l^{-1})	Extract free of sugar (g l^{-1})	Ash (g l^{-1})
	0	11.52	22.18	0	22.18	1.84
Eger,	50	12.11	21.30	0	21.30	1.80
Egri csillagok	200	12.32	21.80	0	21.80	1.82
	800	11.76	22.12	0	22.12	1.97
	1 600	12.22	28.21	0	28.21	2.10
	0	10.30	20.19	0	20.19	1.51
	50	10.72	21.33	0	21.33	1.68
Eger,	200	10.25	21.07	0	21.07	1.61
Olasz rizling	800	10.71	20.24	0	20.24	1.63
	1 600	9.99	20.67	0	20.67	1.73
	0	12.19	18.89	0	18.89	2.57
	50	11.93	17.06	0	17.06	2.17
Miklóstelep,	200	11.17	17.96	0	17.96	2.20
Kocsis Irma	800	10.67	17.50	0	17.50	2.08
	1 600	8.84	77.21	41.04	36.17	3.32
	0	11.31	18.77	0	18.77	1.72
	50	11.44	19.01	0	19.01	1.79
Miklóstelep,	200	11.79	17.68	0	17.68	1.86
Kövidinka	800	10.98	19.50	0	19.50	2.27
	1 600	10.78	21.55	0	21.55	3.05
	0	0	190.40	162.00	28.40	3.02
	50	8.38	22.42	0	22.42	3.58
Miklóstelep,	200	8.46	21.21	0	21.21	2.40
Hárslevelű	800	7.85	28.85	7.51	21.34	2.53
	1 600	8.02	21.27	0	21.27	2.59
	0	10.12	20.89	0	20.89	1.80
	50	9.70	21.10	0	21.10	2.09
Miklóstelep,	200	9.15	24.73	0	24.73	1.93
Hárslevelű 2	800	10.04	20.09	0	20.09	1.80
	1600	9.50	22.43	0	22.43	2.23
	0	11.33	20.63	0	20.63	1.88
	50	10.91	22.76	0	22.76	2.05
Miklóstelep,	200	11.04	21.02	0	21.02	1.89
Hárslevelű 3	800	10.78	20.96	0	20.96	2.45
	1 600	10.69	22.32	0	22.32	3.15

from fruit given radiation treatment

Alkalinity of ash (meq l^{-1})	Titratable acid (g l^{-1})	pH	Free SO_2 (mg l^{-1})	Total SO_2 (mg l^{-1})	Polyphenol index $\left(\frac{A_{280}}{20}\right)$	Colour intensity (A_{420})
22.7	10.7	2.92	5	43	7.60	0.140
21.9	9.2	3.05	4	38	8.90	0.154
21.2	9.0	3.04	5	56	7.56	0.203
22.0	9.5	3.00	5	37	8.20	0.179
23.6	7.8	3.16	7	57	10.06	0.286
21.2	8.5	3.01	2	23	8.20	0.122
22.8	8.5	3.09	3	18	8.40	0.168
21.4	8.6	3.00	3	28	9.72	0.160
20.2	7.9	3.03	4	29	8.42	0.153
21.0	7.4	3.07	5	21	9.44	0.182
30.4	4.6	3.60	5	11	5.70	0.185
26.8	4.1	3.64	4	8	5.58	0.194
26.8	4.8	3.56	5	13	5.56	0.219
26.0	4.5	3.56	5	16	5.96	0.223
33.2	6.4	3.76	8	34	8.72	0.381
21.0	7.4	3.16	7	38	9.00	0.115
23.0	7.9	3.05	5	78	9.24	0.125
22.0	6.6	3.19	6	44	9.00	0.136
26.4	6.0	3.44	5	39	10.40	0.185
34.6	5.9	3.63	3	19	12.20	0.390
35.6	13.2	3.03	7	38	6.58	0.097
32.8	13.2	3.05	3	32	6.18	0.104
28.6	12.5	3.10	5	28	6.18	0.092
27.8	10.8	3.21	7	66	8.72	0.206
35.2	10.3	3.19	4	27	9.40	0.426
23.8	10.6	2.98	6	41	7.40	0.094
28.4	11.6	2.99	8	34	6.10	0.087
24.6	10.8	3.08	9	25	6.25	0.118
25.6	8.6	3.12	7	42	8.04	0.150
27.6	8.8	3.27	10	39	8.82	0.280
24.6	9.7	3.09	10	42	6.58	0.135
26.4	9.6	3.10	10	58	6.80	0.104
24.4	9.0	3.20	13	48	7.36	0.098
28.4	7.7	3.38	11	41	9.10	0.185
35.2	7.4	3.56	8	37	10.00	0.364

3. Conclusions

Grapes treated with 800 and 1 600 suffered a definite change. A brownish discolouration, the stems and skin becoming thinner and softer, in some cases gelatinization or even liquefaction were observed. These phenomena were not noticed in grapes treated with 50 krad. In one or two of the varieties similar phenomena were observed after treatment with 200 krad. Thus the texture of grapes was highly affected by higher doses.

The juice yield of grapes radiation treated increased relative to that of the control sample. In the majority of the experiments, there was a linear correlation between the yield and the square root of the radiation dose. At the highest dose level the yield increase was substantial and amounted to 25—28%.

The process of fermentation appeared unaffected by radiation treatment. Even at higher dose levels the yeasts were not destroyed to such an extent as to inhibit fermentation.

The composition of wine obtained from grapes treated at high dose levels differed from that of the control. Their extract and ash content was generally, their pH always, higher than that of the control. The polyphenol index and colour intensity increased with increasing radiation dose.

The organoleptic quality of wines originating from grapes treated with 800 krad and particularly those treated with 1 600 krad showed a significant and unfavourable change. Their taste and odour was bad, unpleasant, of oxidized character.

From the above the conclusion may be drawn, that though treatment with 800 and 1 600 krad resulted in significantly higher juice yield, their application is not advisable. The composition and organoleptic quality of the wines thus obtained is of very low quality. Upon treatment with 200 krad the juice yield increase is still substantial (7%), while the unfavourable changes are only slight or do not appear at all.

On the basis of these experiences it would seem of interest to repeat the experiments in the range between 200 and 500 krad. Here an increase in yield of importance could be reckonned with and the eventual by-effects could be eliminated by proper treatment of the wines.

It seems essential to carry out industrial experiments, since on industrial presses the juice yield is originally higher than on small laboratory presses.

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Acta Alimentaria, Vol. 4. (2), pp. 167—179 (1975)

FACTORS AFFECTING POLYGALACTURONASE YIELD AND KINETIC TYPES OF ENZYME PRODUCTION BY *ASPERGILLUS AWAMORI*

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(Received February 15, 1974)

Polygalacturonase (PG) formation of *Aspergillus awamori* was studied when grown in 100-litre fermentors. The effect of agitation speed on enzyme synthesis by *Aspergillus awamori* cultivated in sucrose- and pectin-containing media, resp., was investigated.

The increase in the agitation speed from 200 to 300 rpm (with O_2-solution rates of 27 and 85 mmole $l^{-1}\,h^{-1}$) resulted in a very significant (3—4.5-fold) increase in the yields of constitutive endo-PG and apple-juice-clarifying PG, resp., while the yield of the inductive endo-PG increased slightly.

Scaling-up (from 10 to 100 litres) influenced the apple-juice-clarifying PG production of the above strain positively ($SPA^{A}_{75} = 230$ and 500 $l\,h^{-1}\,l^{-1}$, resp.), while the formation of constitutive and inductive endo-PG decreased significantly in the higher scale.

The growth of the cultures was diauxic when cultivated on both carbon sources. Though the mycelial yield increased with increasing speed of agitation, the rates of growth changed to the contrary as a result of the short exponential phases of cultures agitated at lower speed. The growth in these phases was very intensive and the growth rate constant was significantly higher in the second exponential phases at lower speeds.

The increase in agitation speed had a positive effect on the growth-related production rate constant (k_{p1}) only in the case of constitutive endo-PG formation, while it had a negative effect on k_{p1} of inductive endo-PG and apple-juice-clarifying PG. The production rate constants related to non-growing cells (k_{p2}) were found to have measurable values only in cultures agitated at higher speed in the case of constitutive and inductive endo-PG formation, while in the case of apple-juice-clarifying PG it increased from 2.75 to 16.30 h^{-1} with increasing agitation speed.

According to this work the kinetic type of enzyme formation was influenced by the kind of polygalacturonase enzyme component, the speed of agitation and by scaling-up.

Kinetic analysis is an important method not only for the prediction of parameters of continuous fermentation, but also for the evaluation of various parameters of batch fermentation and scaling-up.

Kinetic analysis of the constitutive and inducible polygalacturonase formation of *Aspergillus foetidus* and *Aspergillus awamori* cultivated in 10-litre fermentors was given in a previous paper (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973).

The present work contains the kinetic analysis of the polygalacturonase formation of *Aspergillus awamori* in 100-litre fermentors. The effect of the scaling-up process as well as the influence of oxygen supply on synthesis of

constitutive and inductive endo-PG, apple-juice-clarifying PG and on the kinetic types of enzyme formation, have been studied in the course of cultivation.

1. Materials and methods

An *Aspergillus awamori* strain was grown in 100-litre stainless steel fermentors.

Composition of the media:

I. for *constitutive* enzyme formation: sucrose 70 g, $(NH_4)_2SO_4$ 20 g, KH_2PO_4 2 g, corn-steep-liquor 10 g/1 000 ml, pH 3.5;

II. for *inductive* (inducible) enzyme formation: pectin 30 g, $(NH_4)_2SO_4$ 7 g, KH_2PO_4 2 g, corn-steep liquor 10 g/1 000 ml, pH 4.5. The volume of the media was 80 litres. 8 l of a 24-hour vegetative culture was used as the inoculum. Cultures were aerated at a rate of 1 vol/vol min^{-1} with agitation speeds of 200 and 300 rpm, resp. Oxygen solution rates were 27 and 85 mmole l^{-1} h^{-1} determined by the sulphite oxidation method (Cooper *et al.*, 1944). The temperature was 28 °C and the period of incubation 72 hours.

The mycelial weight of the culture was determined after drying at 105 °C to constant weight.

Determination of activities of enzymes. Endo-polygalacturonase (endo-PG) and apple-juice-clarifying activities of the culture filtrate were measured by a viscometric method (Zetelaki & Vas, 1972).

Substrates used for the determinations: for endo-PG: Na-polypectate (Na-p: Serva, Entwicklungslabor, Heidelberg); and freshly prepared apple-juice (variety Jonathan) with specific viscosity adjusted to 1.0 and pH to 3.8.

Kinetic analysis was carried out with an electronic digital computer using the equations (Kono, 1968; Kono & Asai, 1969) for four-phase growth applied to diauxic growth.

2. Results

Mycelial yield and constitutive endo-PG production of an *Aspergillus awamori* strain as a function of the cultivation time, and the rates of growth and enzyme formation as a function of the mycelial concentration at an agitation speed of 300 rpm, are given in Fig. 1. Data of growth rate, production rates and the kinetic constants are summarized in Table 1.

The growth curve of the culture clearly shows diauxic growth with two transient, exponential and declining phases. The growth rates and the enzyme production rate of the culture at various periods of cultivation plotted against mycelial concentration also proved the existence of the diauxic cycle. The maximum mycelium yields (2.40 g dl^{-1}) and endo-PG production ($SPA_{75}^{Na\text{-}p}$

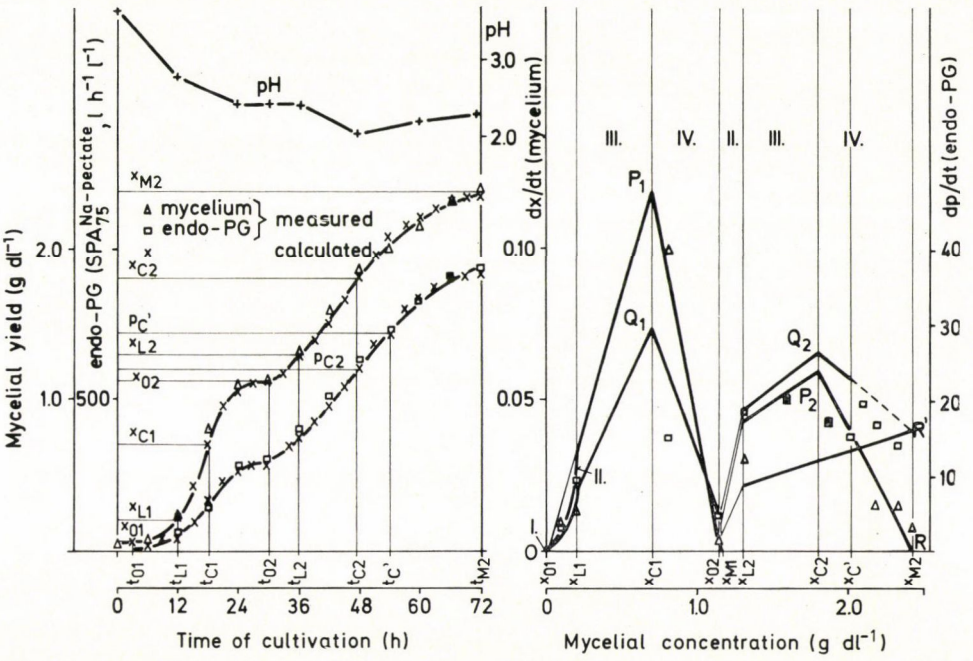

Fig. 1. Formation of mycelium and of constitutive endo-polygalacturonase by *Aspergillus awamori* when grown at an agitation speed of 300 rpm. (Cultivation conditions as in Chapter 1. under *I:* carbon source: sucrose)

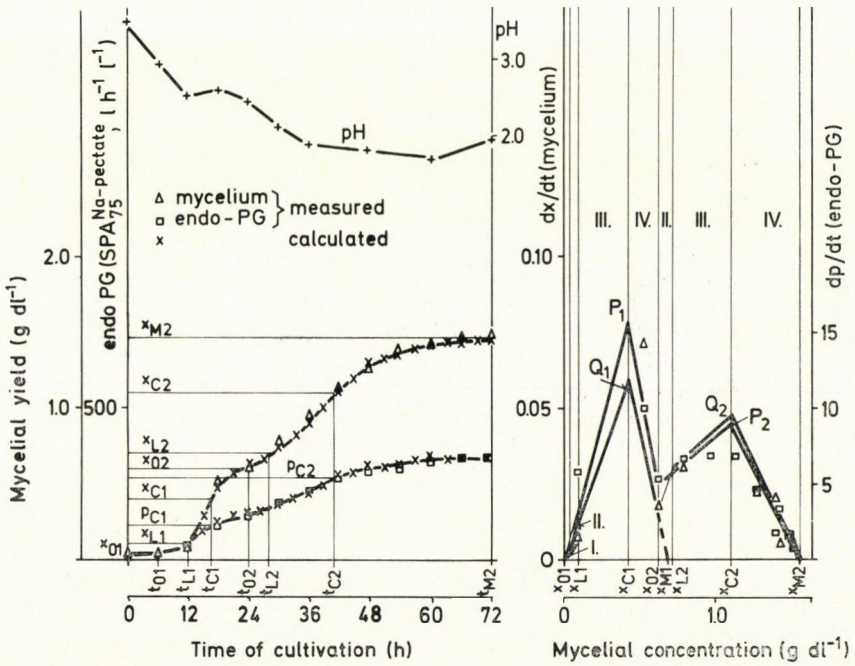

Fig. 2. Formation of mycelium and of constitutive endo-polygalacturonase by *Aspergillus awamori* when grown at an agitation speed of 200 rpm. (Cultivation conditions as in Chapter 1. under *I:* carbon source: sucrose)

Table 1

Characteristic values of constitutive endo-polygalacturonase (endo-PG) formation of Aspergillus awamori culture grown in 100 litre fermentors at different speeds of agitation with no pH control

(Carbon source: sucrose.)

Agitation (rpm)	Diauxic cycle	t_O	t_L	t_C	$t_{M(x)}$	x_O	x_L	x_C	x_M	k
300	1	3.0	12.0	18.0	31.5	0.03	0.20	0.70	1.14	0.172
	2	30.0	34.5	47.5	72.0	1.12	1.25	1.80	2.40	0.033
200	1	6.0	12.0	16.5	25.5	0.03	0.10	0.40	0.65	0.195
	2	24.0	28.0	41.0	72.0	0.60	0.70	1.10	1.47	0.043

Agitation (rpm)	Diauxic cycle	k_{P_1}	k_{P_2}	$k_{P'}$	p_C	$p_{C'}$	p_M
300	1	46.10	0.00	0.000	165	0	0
	2	14.55	7.50	0.127	610	725	935
200	1	30.00	0.00	0.000	120	0	0
	2	8.25	0.00	0.000	260	0	335

Nomenclature

k = growth rate constant, h^{-1}

k_{p1}, k_{p2} = production rate constants

$k_{p'}$ = production rate constant in the final phase of product formation

t = time, h

x = cell concentration, g dl^{-1}

p = product concentration, SPA_{75} l $h^{-1}l^{-1}$

p_c = product concentration belonging to x_c

$p_{c'}$ = product concentration belonging to the beginning of the final phase of product formation

O = refers to the boundary of an induction phase and a transient phase

L = refers to boundary of a transient phase and an exponential growth phase

C = refers to the critical point, that is the boundary of an exponential growth phase and a declining growth phase

M = refers to theoretical maximum value of cell and product concentration, resp.

935 l h^{-1} l^{-1}) were measured in a 72-hour culture. The rate constants of growth in the exponential phase of the first and second diauxic cycle were 0.172 and 0.033 h^{-1}, respectively. The production rate of the endo-PG formation was growth-related in the first cycle (k_{p1} = positive, $k_{p2} = 0$), while in the

second cycle the production of endo-polygalacturonase was the result of the enzyme synthesis of both the growing and non-growing cells.

The value of the production rate constant of the growing cells (k_{p1}) in the first and the second part of the diauxie were 46.10 and 14.55 h^{-1}, resp. while the value of the production rate constant of the resting cells was 7.5 h^{-1}.

The growth and constitutive endo-PG formation as well as the growth and production rates of *Aspergillus awamori* when agitated at 200 rpm were plotted in Fig. 2. Data on growth rate, production rates and the kinetic constants are given also in Table 1.

As seen from the growth curve and from the lines determined by data of rates of growth plotted against mycelial concentration, the growth of the culture was diauxic again. The production rate of endo-PG formation under these less favourable conditions of aeration proved to be growth-related in both parts of the diauxie.

The highest mycelial (1.47 g dl^{-1}) and endo-PG yields ($SPA_{75}^{Na\text{-}p}$ 335 l h^{-1} l^{-1}) were found to be in the 72-hour culture. In the first and second exponential phase of the diauxie the values of the growth rate constants were 0.195 and 0.040 h^{-1}, while the values of the production rate constants were 30 and 8.25 h^{-1}, resp.

The mycelial and inductive endo-PG yields as well as the rate of growth and the enzyme formation of *Aspergillus awamori* culture when agitated at 300 rpm are shown in Fig. 3. Data of the growth rate, production rates and the kinetic constants can be seen in Table 2.

According to the figure, the growth of the strain was also diauxic when cultivated in media containing pectin. The growth and endo-PG formation of the culture was more intensive in the first part of the diauxie than in the second. In the first diauxic cycle the endo-PG synthesis was growth-related, while in the second cycle both the growing and resting cells contributed to the enzyme synthesis. The values of the growth-related production rate constants (k_{p1}) in the first and second exponential phases were 15.38 and 2.86 h^{-1}, resp., while the production-rate constants (k_{p2}) of the resting cells in the first and second declining phases were zero and 1.29 h^{-1}, resp. The values of the growth rate constants in the first and second exponential phases of the diauxie were 0.125 and 0.023 h^{-1}, resp.

The highest mycelial (1.62 g dl^{-1}) and endo-PG yields ($SPA_{75}^{Na\text{-}p}$ 175 l h^{-1} l^{-1}) were obtained in the 60- and 72-hour cultures, resp.

The growth and inductive endo-PG formation of the above culture when agitated at a speed of 200 rpm can be seen in Fig. 4. Data of the growth rate and production rate with the kinetic constants are given also in Table 2.

The culture of the *Aspergillus awamori* strain also showed a diauxic growth when agitated at a lower speed. The maxima of the mycelial and endo-

PG yields of the 66-hour culture were lower (1.48 g dl^{-1} and 126 l h^{-1} l^{-1}) than those of the cultures agitated at a higher speed.

The growth rate constants of the first and second exponential phases were 0.133 and 0.061 h^{-1}, resp. The formation of endo-polygalacturonase was growth-related in both the first and in the second part of the diauxie when the values of the production rate constants (k_{p1}) were 20 and 1.38 h^{-1}, resp.

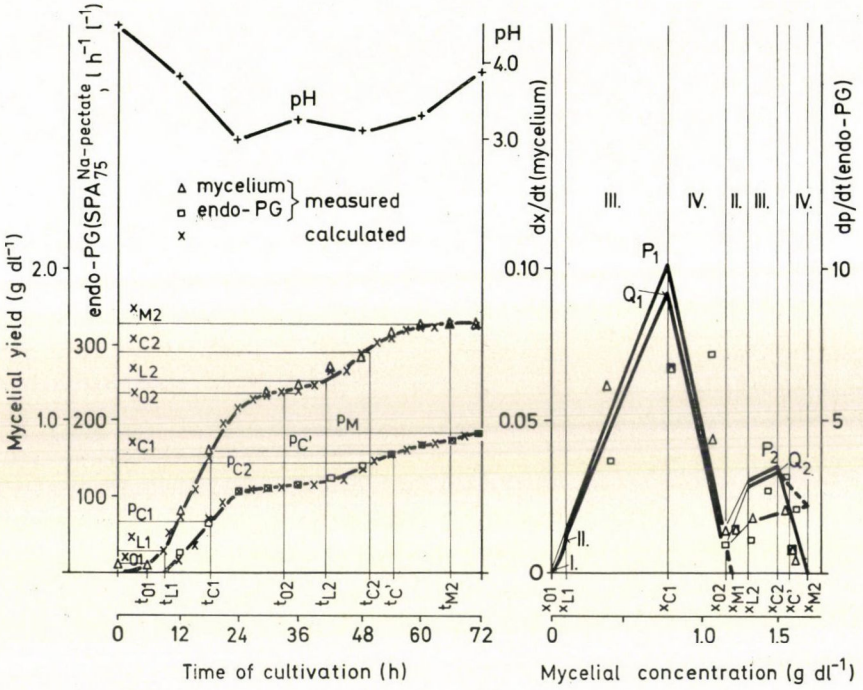

Fig. 3. Formation of mycelium and of inductive endo-polygalacturonase by *Aspergillus awamori* when grown at an agitation speed of 300 rpm. (Cultivation conditions as in Chapter 1. under *II:* carbon source: pectin)

The apple-juice-clarifying component of the polygalacturonase enzyme complex produced by the *Aspergillus awamori* strain proved to be inductive; it was synthetized only when the culture was cultivated in a medium containing pectin.

The growth and production of the apple-juice-clarifying polygalacturonase of the above strain when agitated at a speed of 300 rpm are shown in Fig. 5. Data of growth rate, production rates and the kinetic constants are summarized in Table 3.

The mycelial yield and the rate of growth are the same as given in Fig. 3. The synthesis of the apple-juice-clarifying enzyme component was low

and growth-related in the first part of the diauxic cycle. The synthesis of the above component was rather intensive in the second phase with a high value (16.3 h^{-1}) of the production rate constant of the resting cells (k_{p2}) and a much lower value (4.86 h^{-1}) of the growth-related production rate constant.

The yields of mycelium and apple-juice-clarifying PG of the *Aspergillus awamori* strain cultivated in pectin-containing medium at a speed of agitation

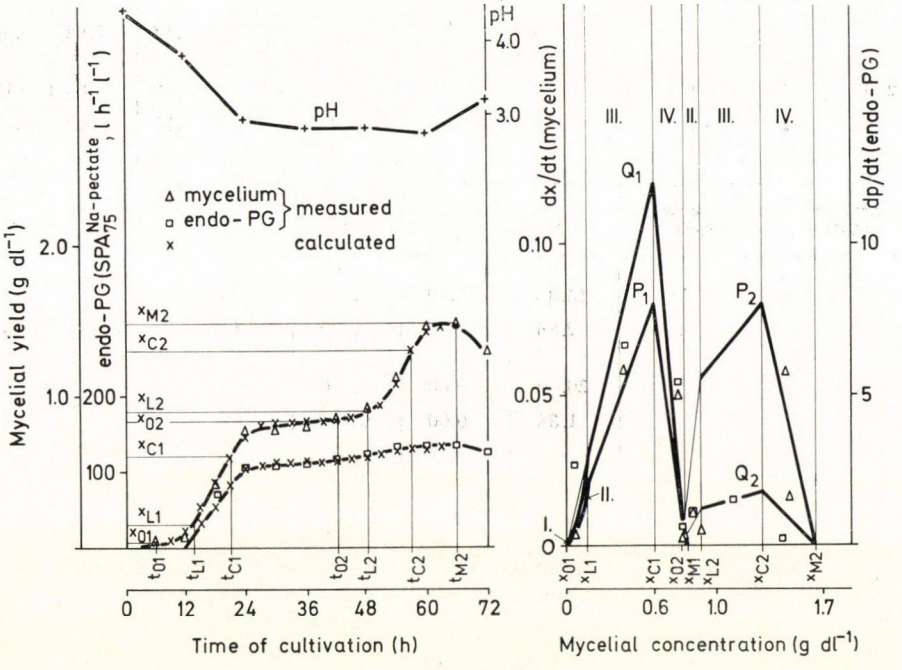

Fig. 4. Formation of mycelium and of inductive endo-polygalacturonase by *Aspergillus awamori* when grown at an agitation speed of 200 rpm. (Cultivation conditions as in Chapter 1. under *II:* carbon source: pectin)

of 200 rpm can be seen in Fig. 6. Data of the growth rate, production rates and the kinetic constants are also given in Table 3.

The growth curve and the growth rates are the same as those in Fig. 4. The formation of the apple-juice-clarifying PG component was very low in the first part of the diauxic cycle and much higher in the second with a maximum yield (SPA_{75}^{A}) of 112 l h^{-1} l^{-1}, measured in the 72-hour culture.

The formation of apple-juice-clarifying PG proved to be growth-related in the first part of the diauxie (($k_{p1} = 5.0$ h^{-1}; $k_{p2} = 0$) while in the second part both growing and non-growing cells contributed to the enzyme formation. The values of the production rate constants of k_{p1} and k_{p2} were 4.66 and 2.70 h^{-1}, resp.

Table 2

Characteristic values of inductive endo-polygalacturonase (endo-PG) formation of Aspergillus awamori culture grown in 100-litre fermentors at different speeds of agitation with no pH control (Carbon source: pectin)

Agitation (rpm)	Diauxic cycle	t_O	t_L	t_C	$t_{M(x)}$	x_O	x_L	x_C	x_M	k
300	1	6.0	9.0	18.0	36.0	0.03	0.15	0.78	1.20	0.128
	2	33.0	41.0	49.0	66.0	1.18	1.26	1.44	1.62	0.021
200	1	6.0	13.5	21.0	45.0	0.03	0.15	0.60	0.83	0.133
	2	42.0	48.0	57.0	66.0	0.82	0.90	1.30	1.48	0.061

Agitation (rpm)	Diauxic cycle	k_{p1}	k_{p2}	$k_{c'}$	p_C	$p_{c'}$	p_M
300	1	15.38	0.00	0.000	65	0	0
	2	2.86	1.29	0.130	145	159	175
200	1	20.00	0.00	0.000	80	0	0
	2	1.38	0.00	0.000	127	0	130

Table 3

Characteristic values of the apple-juice-clarifying PG formation of Aspergillus awamori culture, grown in 100-litre fermentors at different speeds of agitation with no pH control (Carbon source: pectin)

Agitation (rpm)	Diauxic cycle	t_O	t_L	t_C	$t_{M(x)}$	x_O	x_L	x_C	x_M	k
300	1	6.0	9.0	18.0	36.0	0.03	0.15	0.78	1.20	0.128
	2	33.0	41.0	49.0	66.0	1.18	1.26	1.44	1.62	0.021
200	1	6.0	13.5	21.0	45.0	0.03	0.15	0.60	0.83	0.133
	2	42.0	48.5	57.0	66.0	0.82	0.90	1.30	1.48	0.061

Agitation (rpm)	Diauxic cycle	k_{p1}	k_{p2}	$k_{c'}$	p_C	p_C'	p_M
300	1	2.81	0.00	0.000	20	0	0
	2	4.86	16.30	0.129	150	350	500
200	1	5.00	0.00	0.000	17	0	0
	2	4.66	27.5	0.209	72	89	112

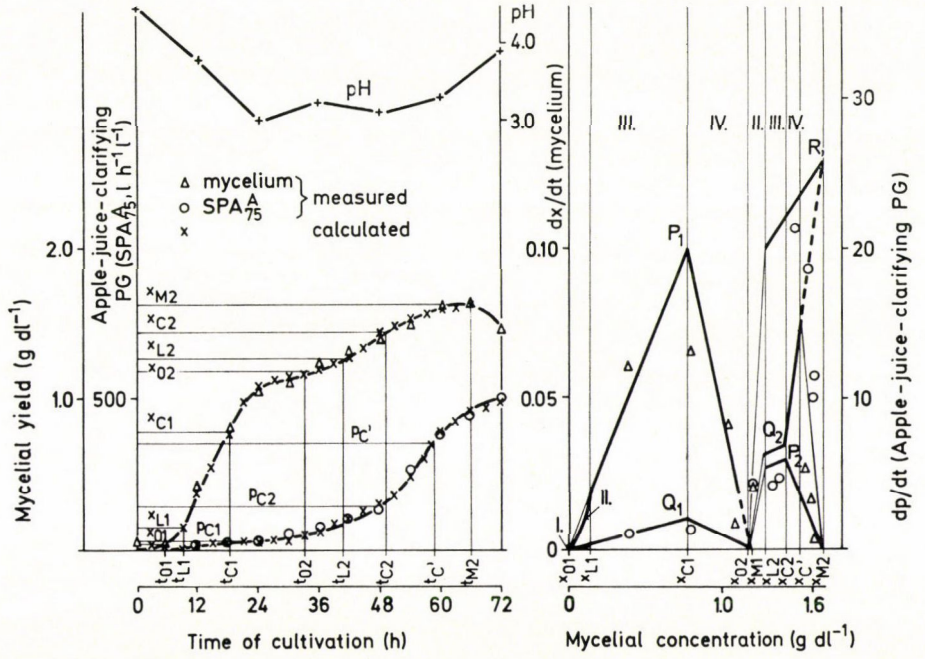

Fig. 5. Formation of mycelium and of apple-juice-clarifying PG by *Aspergillus awamor* when grown at an agitation speed of 300 rpm. (Cultivation conditions as in Chapter 1 under *II:* carbon source: pectin)

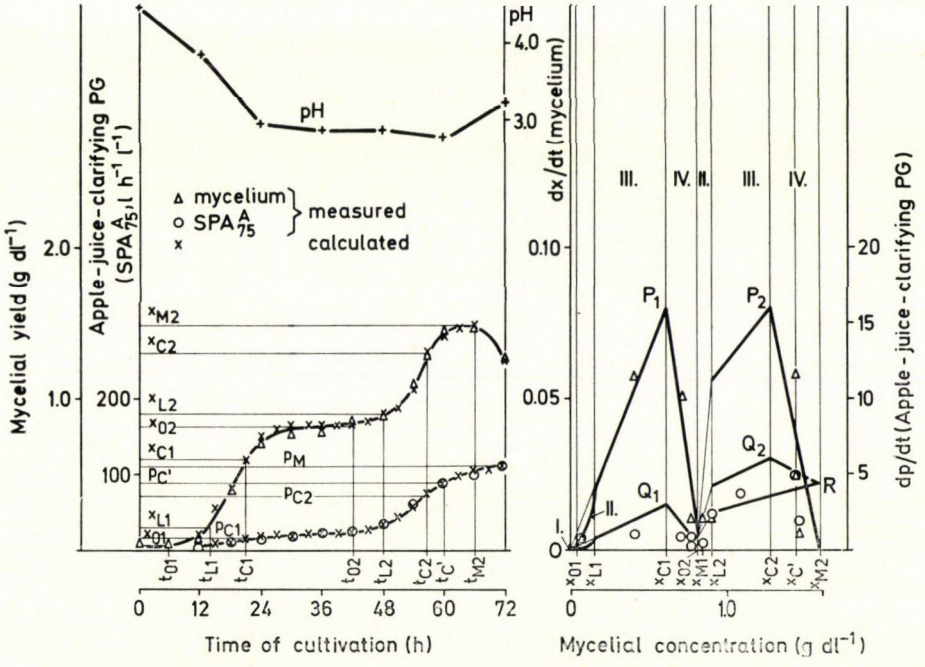

Fig. 6. Formation of mycelium and of apple-juice-clarifying PG by *Aspergillus awamori* when grown at an agitation speed of 200 rpm. (Cultivation conditions as in Chapter 1. under *II:* carbon source: pectin)

3. Conclusions

Data of the present paper and those obtained when the *Aspergillus awamori* strain was cultivated in 10-litre fermentors (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973) are summarized in Table 4.

Table 4

The effect of various conditions of cultivation on polygalacturonase yields (p_M), the production rate constants (k_{p1} k_{p2}) and on the kinetic production type of components of polygalacturonase enzyme complex of Aspergillus awamori

Enzyme component	Carbon source	Volume of fermentors (l)	Dissolved O_2 (mmole l^{-1} h^{-1})	Agitation (rpm)	Diauxic cycle	k_{p1}	k_{p2}	p_M	Type of production
Constitutive		100	85	300	1.	46.10	0.00	—	
					2.	14.55	7.50	935	I.
endo-PG	sucrose	100	27	200	1.	30.00	0.00	—	
					2.	8.25	0.00	335	II.
		10	51	460	1.	47.10	0.00	—	
					2.	18.20	16.65	1 500	I.
Inductive		100	85	300	1.	15.38	0.00	—	
					2.	2.86	1.29	175	I.
endo-PG	pectin	100	27	200	1.	20.00	0.00	—	
					2.	1.38	0.00	130	II.
		10	51	460	1.	107.50	0.00	—	
					2.	98.50	37.50	2 520	I.
Apple-juice-clarifying PG	pectin	100	85	300	1.	2.81	0.00	—	
					2.	4.86	16.30	500	I.
		100	27	200	1.	5.00	0.00	—	
					2.	4.66	2.75	112	I.
		10	51	460	1.	23.00	0.00	—	
					2.	0.00	12.30	230	III.

From a practical point of view it can be established from the results of this experiment that the use of a higher agitation speed (300 rpm with an O_2-dissolution rate of 85 mmole l^{-1} h^{-1}) resulted in higher mycelial and enzyme yields in the 100-l fermentor (p_M in: Table 4). This is in accordance with the results of our previous experiment concerning the effect of agitation on glucose oxidase synthesis (ZETELAKI, 1970). At the above agitation speed (300 rpm) the constitutive endo-PG and the inductive apple-juice-clarifying PG have given about three- and four-fold yields in comparison with the yield of the culture agitated at only 200 rpm (O_2-solution rate: 27 mmole l^{-1} h^{-1}). It had been established previously (ZETELAKI & VAS, 1971) that O_2 dissolution rates higher than 100 and lower than 40 mmole l^{-1} h^{-1} had a negative effect on the formation of polygalacturonases.

Scaling-up, at a higher oxygen supply rate, appeared to have a positive effect on the formation of the apple-juice-clarifying PG of the *Aspergillus awamori* strain, the yield of this component was more than twice that of the product in 10-litre fermentors. A negative effect of scaling-up was found in the production of constitutive and inductive endo-PG. The yields of constitutive and inductive endo-PG synthesized in 10-litre fermentors were 1.5- and 14-times higher than those produced in the 100-litre fermentor at a higher oxygen supply rate (Table 4).

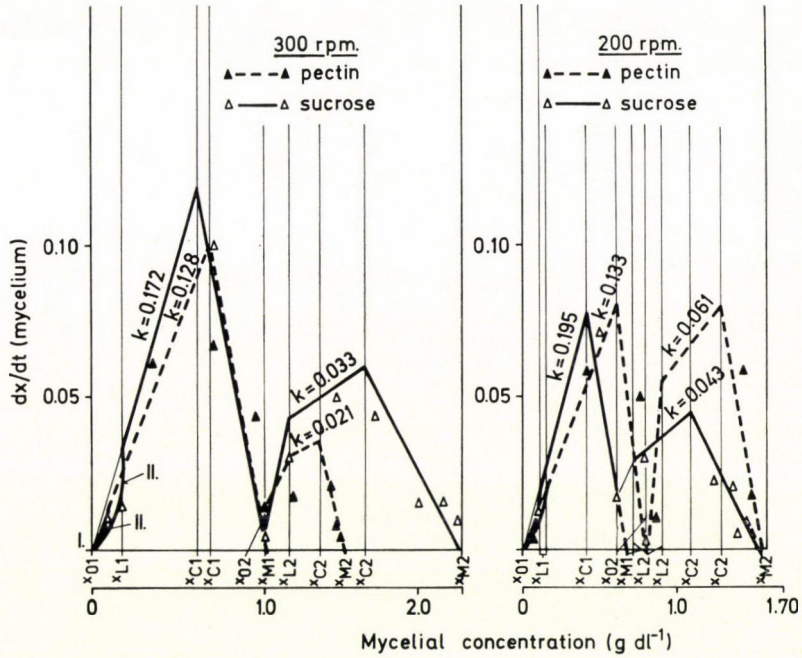

Fig. 7. The rates of growth of *Aspergillus awamori* culture as a function of mycelial concentration in pectin- and sucrose-containing media when agitated at 300 and 200 rpm, resp.

The mycelial yield was not influenced significantly by scaling-up. In pectin-containing media the mycelial yields were about the same at both scales (10- and 100-litre) but in sucrose media the mycelial yield decreased by 21 per cent when cultivated in a larger fermentor.

The kinetic constants were influenced by the above parameters, too. In accordance with our previous paper (ZETELAKI *et al.*, 1973) the growth of the culture was diauxic in both of the media used. The growth rate constants of the *Aspergillus awamori* strain were higher in sucrose media at an agitation speed of 300 rpm, while at 200 rpm the growth rate constants of the culture grown in pectin media were higher (Fig. 7).

The values of the growth-rate constants (k) were higher in cultures agitated at lower (200 rpm) speeds (Table 2). This can be explained by the characteristics of the growth curves of the cultures at different speeds of agitation. The cultures agitated at a lower speed have very short exponential phases with intensive growth even in the second part of the diauxie. The cultures agitated at higher speeds have produced about 50 and 70 per cent of their mycelial yield in the first part of diauxie when incubated in sucrose and in pectin media, resp.

The values of the growth-related production rate constants (k_{p1}) did not change uniformly under various agitation conditions. Increase of the agitation speed from 200 to 300 rpm. resulted in an increase of the k_{p1} only in the case of the constitutive endo-PG. The production rate constants of the non-growing cells (k_{p2}) were zero when cultures were agitated at a lower speed, and had a rather significant value at a higher speed of agitation in the case of constitutive and inductive endo-PG.

In the case of apple-juice-clarifying PG the value of k_{p2} of the culture agitated at 300 rpm was about six-fold that of the culture agitated at the lower speed (Table 4).

According to the present work, the kinetic type of enzyme formation was influenced by the following: 1. kind of the polygalacturonase enzyme component, 2. agitation speed and 3. scaling-up (Table 1.).

KONO and ASAI (1969) determined the types of product formation of the microorganism in the case of the simple four-phase growth. It was established in a previous work (ZETELAKI, 1972) that in the case of diauxic growth the types of product formation are determined by the values of the production rate constants of the second part of diauxic growth.

1. The production types of components of the polygalacturonase enzyme were not the same under identical conditions of cultivation. In cultures agitated at the lower speed of 200 rpm, the formation of constitutive and inductive polygalacturonase was growth-related (type II: $k_{p1} = +$ and $k_{p2} = 0$) while the formation of apple-juice-clarifying PG was the result of the enzyme synthesis of both the growing and non-growing cells as well (type I: $k_{p1} = +$ and $k_{p2} = +$).

2. In the case of enzyme synthesis of constitutive endo-PG the increase of the agitation speed altered the type of product formation. Under a condition of higher oxygen supply not only the growing (type II) but the non-growing cells took part in the enzyme synthesis (type I of product formation).

3. Scaling-up also had an influence on the production types of the enzyme. The apple-juice-clarifying PG was synthesized by non-growing cells (type III: $k_{p1} = 0$ and $k_{p2} = +$) when cultures were incubated in 10-litre fermentors, while in 100-litre fermentors the above component was produced by both the growing and non-growing cells (type I: $k_{p1} = +$ and $k_{p2} = +$).

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Acta Alimentaria, Vol. 4. (2), pp. 181–188 (1975)

KINETIC ANALYSIS OF PROTEIN SYNTHESIS IN FUNGI

PART I.—THE EFFECT OF COMPOSITION OF MEDIA ON THE GROWTH AND PROTEIN FORMATION OF *ACTINOMUCOR REPENS*

K. ZETELAKI-HORVÁTH, K. VAS and A. ABD EL-BAKEY MAHMOUD

(Received March 25, 1974)

Growth and protein formation of an *Actinomucor repens* (Strain No. 207) culture under submerged conditions in agitated and aerated fermenters were studied in culture media of different composition. The growth of the above strain was negligible when synthetic glucose and sucrose media were used. The completion of the media with corn-steep liquor resulted in a remarkable growth response. Maximal mycelial and protein yields of the culture in glucose and in sucrose media containing corn-steep liquor were 8.4 and 8.7 for mycelial yield and 4.1 and 3.5 g l^{-1}, resp., for protein yield. The growth rate constant (k) of the *Actinomucor* culture grown in semi-synthetic sucrose medium was much higher (0.83 h^{-1}) than in media containing glucose (0.58 h^{-1}). The production rate constants (k_{p1}) of protein formation proved to be equal (0.33) in both of the above media used. When a medium, containing hydrolysed bread as a carbon source, was used, the mycelial and protein yields increased more than 2.5-fold of the above yields (22.0 and 10.7 g l^{-1}, resp.). The rate constants of growth and protein formation of the culture grown in the above natural media were also significantly higher (1.30 and 0.79 h^{-1}) than the ones in the semi-synthetic media.

The rapid increase in the population of the earth makes it necessary to produce about 1.3 M t more protein each year (GRAY, 1966). Such an expansion of the protein production cannot be based on agriculture alone. This is the reason why microbial protein as a new source of protein is of current interest for animal feeding as well as for human nutrition.

Fungi form one group of microbes upon which commercial food synthesis can be based. They show great efficiency in using energy from cheap carbohydrates to convert elementary nutrients into protein. Their filamentous structure, together with their rich enzyme systems, make it possible for them to utilize waste products without previous artificial (chemical) hydrolysis.

In the present work the mycelium and protein production of a strain of the fungus *Actinomucor repens* was examined under submerged conditions when glucose, sucrose and bread were used as the carbon source in the media. In spite of the fact, that ground bread can thoroughly be utilized by the culture, bread was added to the media *after hydrolysis* to avoid the errors in the measurement of mycelial yield caused by undigested bread particles in the early stages of the growth. The growth and protein synthesis were kinetically analyzed by the method of KONO and ASAI (1968) and kinetic constants of the above strain grown in various media were compared.

1. Materials and methods

1.1. Microorganism

An *Actinomucor repens* strain (No. 207) was used as the test organism (from the Strain Collection of the *Research Institute of Viticulture and Oenology*, Budapest). Stock cultures were maintained on agar slants. The spore suspension used as the inoculum contained 10^6 conidia per ml of water.

1.2. Nutrient media

The composition of the *inoculation medium* was as follows: yeast 5.0 g (in the form of an extract); soluble starch 15 g; KH_2PO_4 1.0 g and $MgSO_4 \cdot 7H_2O$ 0.5 g adjusted to 1 000 ml with tap water; pH 5.5.

Synthetic (**Nos. 1** and **3**), semi-synthetic (**Nos. 2** and **4**) and natural media (**No. 5**) were used for fermentation. The organic matter sources consisted of **No. 1**: glucose 80 g; **No. 2**: glucose 80 g, corn-steep liquor 20 g; **No. 3**: sucrose 80 g; **No. 4**: sucrose 80 g, corn-steep liquor 20 g; **No. 5**: ground bread 40 g; corn-steep liquor 20 g. (400 g of dry ground bread were suspended in 9 400 ml of tap water. This was heated to 80 °C, then 16 ml of cc. H_2SO_4 were added. The hydrolysis was carried out at 120 °C for 60 minutes). — After hydrolysis the pH was adjusted to 4.0 with NaOH; mineral salts were incorporated in each of the above media as follows: $(NH_4)_2SO_4$ 10 g; $ZnSO_4$ 0.25 g; $MnSO_4$ 0.10 g; KH_2PO_4 1.0 g; volume was adjusted to 1 000 ml with tap water, pH to 4.0.

1.3. Method of cultivation

100 ml of the inoculation medium were inoculated with 1 ml of a spore suspension and were incubated for 24 hours on a rotary shaker (rpm: 330; stroke: 20 mm; oxygen transfer rate in the flasks: 17—19 mmole l^{-1} h^{-1} at a temperature of 28 °C). The fermentations were carried out in 10-litre glass fermenters. The 6 000 ml of medium contained in a fermenter were inoculated with 600 ml of the above 24-hour vegetative culture. The speed of the agitation was 460 rpm, and the volume of air that was bubbled through the culture media was 1.0 l l^{-1} min^{-1}. The oxygen transfer rate in the fermentor was 50.8 mmole l^{-1} h^{-1} as determined by the method of COOPER *et al.*, (1944).

1.4. Preparation of mycelia

Mycelia were obtained from the fermentation broth by filtration through a Nylon cloth (mesh: 60 μm). The residues of the medium were removed by thorough washing with water, then the mycelia were dried at 105 °C to constant weight.

1.5. Protein determination

The protein content of the mycelia was determined by the *Biuret* method as modified for protein determination of whole cells by HERBERT *et al.* (1971a).

1.6. Carbohydrate determination

Reducing sugars were measured by the method of SOMOGYI (1952), while the total carbohydrate content of the fermentation broths was determined by the phenol method (HERBERT *et al.*, 1971b).

1.7. Kinetic analysis

Growth and protein formation of the culture was analysed kinetically. With the use of the kinetic constants determined graphically, mycelial and protein yields were calculated by the equations given by KONO (1968) and KONO and ASAI (1968) for the determination of growth and product concentration of the cultures, respectively.

Kinetic analysis of diauxic growth was performed by the method of KONO and ASAI (1971) and that of product (protein) formation according to ZETELAKI-HORVÁTH (1972).

2. Results

Mycelial and protein synthesis of the *Actinomucor repens* strain were examined under submerged conditions in culture media of different composition.

At first, cultivation of the *Actinomucor repens* strain was attempted in glucose and sucrose containing synthetic media. A very slow rate of growth could be observed when the above strain was incubated in synthetic media (media **Nos. 1** and **3**, in para: 1.2.). The mycelial yields were not sufficient to give a regular growth curve. The above media were then completed with corn-steep liquor. The mycelial and protein yields and data of rates of growth and protein formation of the *Actinomucor* culture in a medium containing glucose and corn-steep liquor (medium **No. 2**) are given in Fig. 1 and Table 1.

The mould consumed 4.0 per cent sugar until maximum mycelial yield was obtained. The pH of the culture filtrate showed a decreasing tendency in the transient and in the exponential phases and an increase in pH value was observed in the period of the autolysis.

The growth of the *Actinomucor repens* strain followed the pattern of a regular 4-phase growth curve. The rate constants of growth and protein formation were 0.58 and 0.33 h^{-1}, resp. Maximum mycelial and protein yields (8.4 and 4.1 g l^{-1}, resp.) were attained in the 42-hour culture.

Table 1

Kinetic constants of the Actinomucor repens strain, grown in semi-synthetic and natural media

Media	t_O	t_L	t_C	$t_{M(x)}$	x_O	x_L	x_C
No. 2. glucose + corn-steep	6	12.0	28.0	42	0	1.0	6.0
No. 4. sucrose + corn-steep	6	15.0	31.5	42	0	1.0	6.0
No. 5. bread	3	7.5	14.0	48	0	2.0	8.8

	x_M	k	k_{P_1}	k_{P_2}	p_C	p_M	$t_{M(p)}$
No. 2. glucose + corn-steep	8.4	0.58	0.33	0	0.33	4.1	42
No. 4. sucrose + corn-steep	8.7	0.83	0.33	0	0.26	3.5	42
No. 5. bread	22.0	1.30	0.79	0	4.80	10.7	48

t_M and $t_{M(p)}$ time of the maximum mycelium and protein yields in hours (data of yields are related to g l^{-1})

The mycelial and protein yields, as well as the rates of growth and protein formation of the *Actinomucor repens* strain in culture media containing sucrose and corn-steep liquor (medium **No. 4**) are given in Fig. 2 and Table 1.

As can be seen in Fig. 2 the pH-value and the sugar content of the fermentation broth decreased until the 42nd hour of the fermentation. In the following period when the autolysis of the mycelia started the pH value of the culture filtrate showed an increasing tendency.

Maximal mycelium and protein yields were obtained at 42 hours of cultivation (8.7. and 3.5 g l^{-1}, resp.). The growth curve of the culture consisted of four phases. The rate constants of growth and of protein formation of the culture proved to be 0.83 and 0.33 h^{-1}, resp.

Since the completion of the culture media of *Actinomucor repens* with corn-steep liquor resulted in an increased rate of growth, the effect of natural media containing bread and corn-steep liquor (medium **No. 5**) was also examined. The mycelial and protein yields, the rates of growth and protein formation of the *Actinomucor* culture in bread medium are given in Fig. 3 and in Table 1.

The pH of the fermentation broth showed a slight decrease (from pH 4.0 to 3.45) until maximum growth was attained, and an increase afterwards

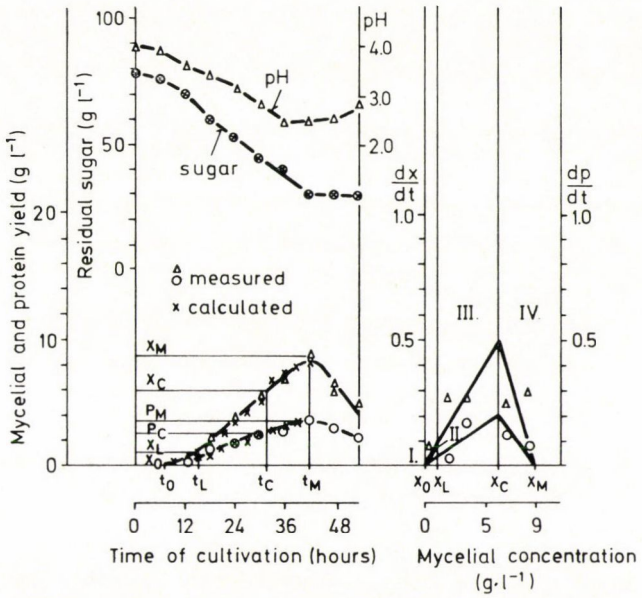

Fig. 1. Mycelial and protein yields and rates of growth and protein formation of the *Actinomucor repens* (No. 207) culture grown in semi-synthetic glucose (**No. 2**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-transfer rate: 50.8 mmole l^{-1} h^{-1})

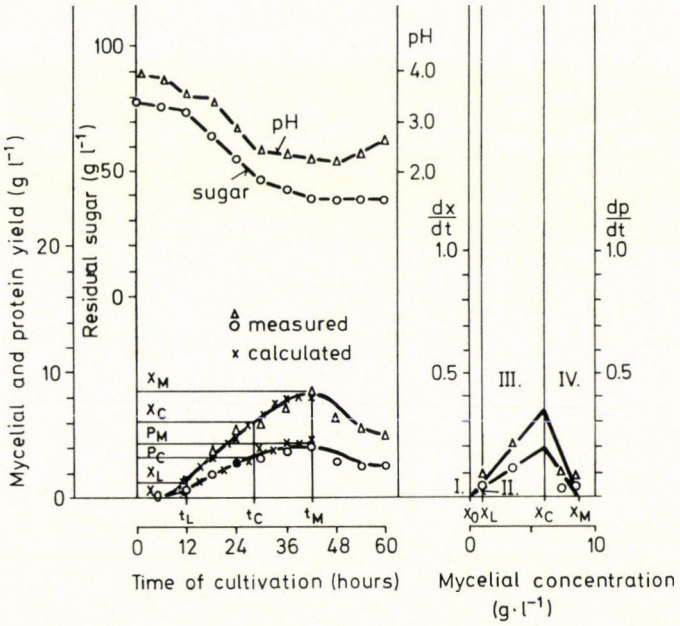

Fig. 2. Mycelial and protein yields and rates of growth and protein formation of the *Actinomucor repens* (No. 207) culture grown in semi-synthetic sucrose (**No. 4**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-transfer rate: 50.8 mmole l^{-1} h^{-1})

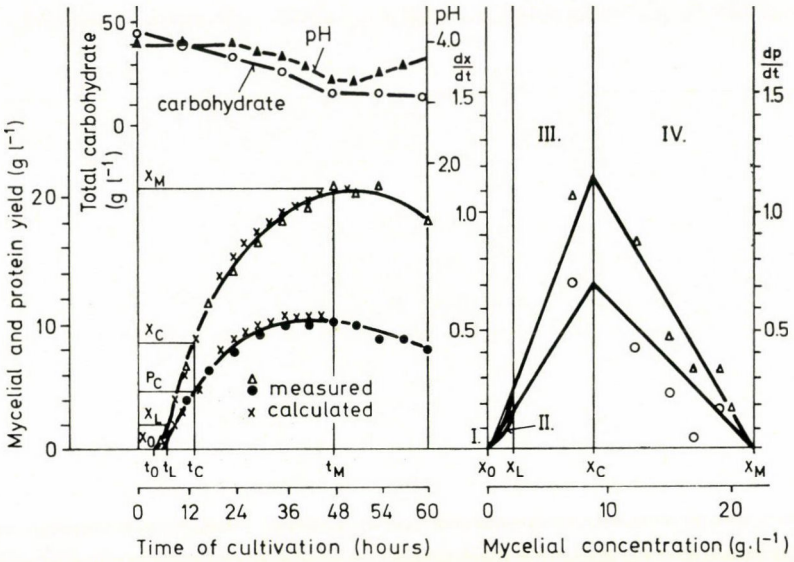

Fig. 3. Mycelial and protein yields and rates of growth and protein formation of the *Actinomucor repens* (No. 207) culture grown in hydrolysed bread (**No. 5**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-transfer rate: 50.8 mmole l^{-1} h^{-1})

toward the end of the fermentation. The 3.5 per cent initial carbohydrate content of the media decreased by 1.6 per cent until the 48th hour of the fermentation.

Maximum mycelial and protein yields (22.0 and 10.7 g l^{-1}, resp.) were attained in the 48-hour culture. The rate constants of growth and protein formation of the culture were found to be 1.30 and 0.79 h^{-1}, resp.

3. Conclusions

In our previous work diauxic growth of various *Aspergillus* strains was found to take place when cultures were grown in media containing sucrose and corn-steep liquor (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973; ZETELAKI-HORVÁTH *et al.*, 1973). The rate of growth and protein synthesis of *Actinomucor repens*, *Rhizopus cohnii* (ZETELAKI-HORVÁTH *et al.*, 1974a) and *Mucor mucedo* (ZETELAKI-HORVÁTH *et al.*, 1974b) strains were also studied. When the *Actinomucor repens* strain was cultivated in glucose and sucrose media containing corn-steep liquor, diauxic growth was not observed. The cultures followed the pattern of a simple one-stage growth curve consisting of four phases.

From the fact that insufficient growth of the above strain was obtained in synthetic media and the mycelial yield could significantly be increased by

complementing the above media with corn-steep liquor, the conclusion can be drawn that culture media must be supplemented with natural components in order to get a good cell synthesis.

Maximum mycelial yields (8.4 and 8.7 g l^{-1}, resp.) were attained in glucose and sucrose media complemented with corn-steep liquor when the age of the culture was 42 hours. Maximum protein yields were also obtained in cultures of the same age but in contrast to the mycelial yields, the maximal protein yield was higher in glucose (4.1 g l^{-1}), than in sucrose (3.5 g l^{-1}) media ($P \geq 95\%$, $r = 0.89$). The growth rate constant of the culture in the exponential growth phase was higher (0.83 h^{-1}) in media containing sucrose than in that containing glucose (0.58 h^{-1}), while the protein production rate constants were equal (0.33 h^{-1}) in both of the above media. The mycelial (22.0 g l^{-1}) and the protein yields (10.7 g l^{-1}) of the *Actinomucor repens* culture in bread hydrolysate were more than two and a half times higher than the yields obtained in the above glucose and sucrose media. This result is of great practical importance since the large amount of dry bread being available as waste product in the households and in the baking industry could be economically utilized for protein production by fungi.

The values of the growth rate and production rate constants were significantly higher (1.30 and 0.79 h^{-1}) in this natural medium than in the above semi-synthetic media.

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Acta Alimentaria, Vol. 4. (2), pp. 189—197 (1975)

EFFECTS OF ZEARALENONE AND SOME DERIVATIVES ON ANIMALS FED ON CONTAMINATED FODDER

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(Received July 8, 1974)

The mycotoxin content of maize damaged by *Fusarium* moulds was investigated. After extraction with chloroform the toxic components were purified and separated with the aid of column and thin-layer chromatography. The method of *Mirocha* was used. The total toxin content of maize varied between 70—80 ppm. In addition to the toxic components described by *Mirocha* a new component — having an R_f value of 0.41 — was separated. The new compound was isolated and investigated by IR spectrophotometry. Some differences in IR spectra were observed related to that of zearalenone.

Pigs, rabbits and deers were fed the mycotoxin-containing maize. The toxin content of different organs of the fed animals was examined. The liver and the gastric content contained in all cases zearalenone. Oestrogenic effect of the mycotoxins was observed in all cases. No toxic compounds occurred in the genitals.

The new ($R_f = 0.41$) component of the toxic substances was prepared in crystalline form and further investigations will be made to elucidate the chemical composition and biological effect of this compound.

From phytopathological aspects, *Fusarium* is one of the most significant genera. Recently a number of *Fusarium* species caused severe economical damage almost throughout the world, either in plant breeding, due to phytotoxic effect or in animal husbandry, due to zootoxic effect.

Early in the preliminary stage of the toxicological research of *Fusarium* species, the above biologically active materials were classified by toxic effects. Several toxin groups were established, corresponding to syndromes observed in animals, like "oestrogenic factor" or "emetic factor". By that time, no pathogenic agents were isolated, simply diseases coincident with the presence of *Fusarium* were demonstrated.

The research of oestrogenic metabolites has been launched in the last few decades (MIROCHA *et al.*, 1967), throwing light on several fundamental facts. These showed several perfect forms of *Gibberella zeae* to develop metabolite likely to cause oestrogenic symptoms in various animal species, such as vulval swelling, vaginal prolapse and hypertrophy of mammalian glands in young sows, and enlargement of the uterus in young, female rats. Hungarian researchers stated the toxin of *Fusarium graminearum* to cause pseudo-oestration. PALYUSIK (1972) observed harmful toxic effects on the spermatogeny of ganders.

Basic results of toxicological research with *Fusarium graminearum* were published by MIROCHA and CHRISTENSEN (1965) stating that a strain growing on the autoclaved corn produces oestrogenic toxins *F-1*, *F-2* and *F-3*. *F-1* was shown to be identical with ergosterol, while *F-2* to be the enanthiomorph of oestrogen, chemically described as 6-(10-hydroxy-6-oxo-*trans*-1-undecyl)-β-resorcyl acid lactone. The above authors will continue their research by determining the chemical nature of *F-3*.

Available results justify the statement that the toxin probably responsible for the sterility of cattle is rather similar to toxin *F-2*, recently called zearalenone. This similarity is apparent from the release of *F-3* from mycelia in obtaining zearalenone chemically, and it can well be detected beside *F-2* with the same reagent and the same developing method.

Tests with the *Fusarium graminearum* toxin led to the statement of the decisive importance of zearalenone (toxin *F-2*) for the toxicity of maize contaminated with *Fusarium*.

Maize infected with *Fusarium graminearum* may not be toxic to animals. In such cases, however, the presence of zearalenone could not be chemically detected, either. This surprising observation is explained by tests connected with the optimum conditions of zearalenone biosynthesis (PRENTICE & DICKSON, 1968). Earlier observations showed *Fusarium graminearum* to have produced toxin only if exposed to low temperature at a given incubation stage. The above authors based their tests on this observation, and grew fungus cultures on sterile maize for different periods incubated at different temperatures, then studied the rates of biosynthesis of zearalenone and ergosterol. Cultures being incubated at 12 °C for three weeks produced 3 500 ppm of zearalenone but no ergosterine. At 25 °C, under otherwise identical conditions, toxin production of fungus strains shifted towards ergosterol while no zearalenone was found. Subsequent test data showed a low temperature period necessary to be included in the incubation time, making the fungi able to produce zearalenone subsequently at higher temperatures. Of the temperatures of 12, 27 and 32 °C, 12 °C is optimal for toxin production. With increasing incubation time toxin production grows.

Thus, temperature and incubation time can be stated to significantly affect zearalenone production.

Composition of the medium often determines physiological and morphological variability of microorganisms, a fact to remember in connection with *Fusaria*. To our best knowledge, no studies on the relation between toxin production of *Fusarium* and medium composition have been made so far. Some newer results (CALDWELL *et al.*, 1970) are of interest which demonstrate that of several *Fusarium* species only *F. graminearum* produces zearalenone.

The authors isolated several *Fusarium* species and grew them on autoclaved maize. After incubation at 16 °C for three weeks, cultures were extracted

by absolute ethanol. Extracts were demonstrated by thin-layer chromatography to contain zearalenone in the following strains: *F. roseum "Culmorum"*, *F. roseum "Equiseti"*, *F. roseum "Gibbosum"*, *F. roseum "Graminearum"* and *F. tricinctum*. It should be mentioned that no toxin production was observed under the same conditions for other *Fusaria: F. moniliforme, F. nivale, F. oxisporum, F. solani.*

Also data contradictory to those by the authors were referred to have been published on *F. moniliforme*. Strains of the latter were found to produce the metabolite *F-3*. Even MIROCHA and co-workers (1967) demonstrated that this strain produced important quantities of zearalenone (8 μg of toxin per g of maize).

Other research workers (WÖLLER & BIRÓ-GOSZTONYI, 1971) showed that *F. culmorum* produced zearalenone in certain circumstances.

In the years 1971 to 1973, *Fusarium* contamination caused important losses in Hungarian agriculture. As a consequence of a cool, rainy autumn weather, 45.5 per cent of the 1972 maize crop exhibited microbiological or toxicological contamination (ETTER, 1973). Fodder made of maize infected with *Fusarium* fungi often caused oestrogenic syndroms in pigs and other domestic animals. According to recent observations (KARNER, 1974) reduced proliferation of hares can be attributed to *Fusarium* toxins. Death of game (deer, stag, fallow deer) visiting agricultural areas has been reported of, and attributed to zearalenone toxin and derivatives.

Zearalenone and its derivatives extracted from maize collected from different crop lands have been tested. Animal tests have been made to see in what organs zearalenone and derivatives of oestrogenic effect can be demonstrated, further if zearalenone derivatives alone produce the same symptoms in the organism as zearalenone itself.

1. Materials and methods

1.1. Extraction of zearalenone

Maize fed at pig-farms causing fusario-toxicosis was applied to obtain zearalenone toxin. Each time 50 g maize was exposed to 6 h of chloroform extraction in a *Soxhlet* extractor to prepare toxin. Chloroform was removed from the oil phase obtained in a rotary vacuum distillation evaporator flask. The residue was purified by the method of MIROCHA and co-workers (1968b) by extraction and chromatographical methods.

1.2. Testing of purified extracts by thin-layer chromatography

The extract prepared by the methods described in paras. 1.1. and 1.5. was tested by thin-layer chromatography.

The thin-layer plate was made (according to *Stahl*) from a mixture of 4 g of Kieselgel-G, and 8 g of water, uniformly applied on a 20 by 20 cm glass plate. After the plate was dried, the gel was activated in a desiccator at 105 °C±2.

Of the standard alcohol solution of *F-2*, 2, 4 and 6 μl, resp., corresponding to 1, 2 and 3 μg of toxin, were applied to the plate according to *Mirocha*'s method. At 2-cm spacings, 5, 10 and 20 μl chloroform stock solutions were applied, obtained from the extract of viscera, and 5 μl of the toxin mixture from the fodder maize extract.

To run the thin-layer chromatogram, a 90 : 45 : 5 mixture of toluene, ethyl acetate and formic acid was applied. After 60 min of development the solvent residue was removed from the plate by hot-air drying. The developed chromatogram was examined in UV light (385 nm) and the toxin *F-2* found to emit a greenish fluorescence at 0.78 R_f, while toxins *F-3*, and *F-4* at 0.70 and 0.65 R_f, respectively. The test material contained toxin *F-2* at identical R_f values, in addition to an UV-active spot at 0.45 R_f, probably due to toxin decomposition. Semi-quantitative evaluation was based on spot area and intensity.

1.3. Testing of purified extracts with gas chromatography

Zearalenone and derivatives were also tested by gas chromatography. 0.5 ml of extract purified chromatographically was transformed into trimethyl silyl ether by the *Shotwell* method (SHOTWELL *et al.*, 1970: 0.1 ml of extract + 0.1 of pyridine + 0.1 ml of chlorine trimethyl silane + 0.2 ml of hexamethyl trisilazane). Chromatography was performed in a HEWLETT-PACKARD type gas chromatograph by flame ionization detection, on a 150 cm long column, composed of 5% separation fluid SE-30, and Chromosorb W 60/80 carrier. Temperatures of column and evaporating chamber were 238 °C and 280 °C, resp., nitrogen was applied as a carrier gas (25 ml min^{-1}).

1.4. Analysis of the IR spectra

For a detailed analysis of the compounds, their IR spectra were examined, using a ZEISS spectrometer type UR-20.

1.5. Examination of the biological activity of toxins

Biological activity of toxins *F-2*, *F-3* and *F-4* was studied in animal tests. A fifty-fifty mixture of maize infested with *Fusarium graminearum* and *F. culmorum* was used to produce a fodder containing 50 γ kg^{-1} zearalenone.

14-day feeding tests were made with albino rats, rabbits and pigs.

In the course of the feeding tests, the daily toxin dosage for the different animal species was adjusted by fodder batching. Zearalenone and derivatives were administered to albino rats, rabbits and pigs in quantities of 5γ, 10γ and 50γ, respectively. Toxin accumulation in the organism was tested on three animals of each kind, and at each time. A control group was fed on toxin-free maize. After the 14-day test period, animals were dissected and toxin contained in liver, uterus, testicles and gastric content examined. The same was done for dead animals.

Viscera (liver, uterus, testicles) have been cut to 0.25 to 0.5 cm pieces and homogenized in a *Biomix* disintegrator in a chloroform solution. The homogeneous material was treated for 6 h in a *Soxhlet* apparatus to extract toxin *F-2*. After extraction, the chloroform solution was cooled to -70 °C with dry ice and frozen fats removed. Chloroform was removed by distillation, the residue was washed 3 times in 10 ml petroleum ether each, to remove traces of fat.

The residue was dissolved in 1 ml of chloroform and its *F-2* content tested by thin-layer chromatography (See para.1.2.).

2. Results

Zearalenone could invariably be detected from the toxophorous maize by thin-layer chromatography at R_f 0.78 by its UV absorption. At lower R_f values different from that of zearalenone *i.e.* at 0.70, 0.65, 0.60, again spots of greenish fluorescence were detected, at decreasing concentrations corresponding to the decreasing R_f values. To improve detection, thin-layer toxin separation was repeated with two-dimensional development. In the first direction, the quoted toluene – ethyl acetate – formic acid mixture was applied, in the other one, 85 : 10 : 5 mixture of benzene – ethyl acetate – acetic acid was applied where R_f 0.42 was obtained for zearalenone and R_f 0.41 for compound *F-4*. Two-dimensional separation gave eight distinct but adjacent UV-active spots. A typical chromatogram is shown in Fig. 1.

At 2.7 min retention time, the gas-chromatogram exhibited a peak identified as zearalenone. Two minor peaks appeared at 3.1 and 3.4 min.

Comparison between gas chromatography and thin-layer chromatography tests showed the demonstrability of only three peaks by gas-chromato-

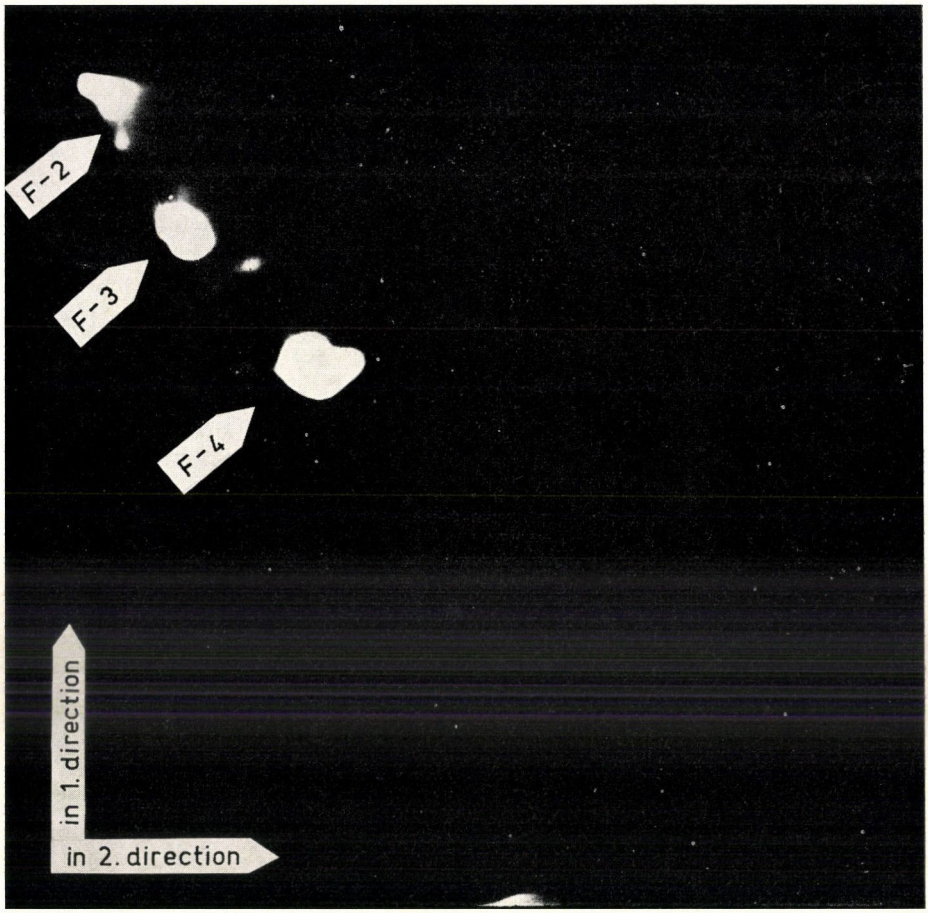

Fig. 1. Typical two-dimensional thin-layer chromatogram of *Fusarium* toxins (Kieselgel G, toluene—ethyl acetate—formic acid 90 : 45 : 5 and benzene—ethyl acetate—acetic acid 85 : 10 : 5)

graphy from the eight various spots, indicating either very low quantities of derivatives of zearalenone or the various compounds to exhibit structural similarities preventing their separation.

In further tests, from the eight UV-active compounds isolated by thin-layer chromatography, three were eluted from the silica gel layer by absolute ethanol, corresponding to R_f values of 0.78 (0.42), 0.70 (0.42) and 0.65 (0.41) resp., designated conventionally as *F-2*, *F-3* and *F-4*.

On the basis of IR spectra typical absorption maxima of toxin *F-2* appeared at wave numbers: 3 400, 2 950, 2 935, 2 860, 1 690, 1 650, 1 618, 1 580, 1 450, 1 430, 1 390, 1 360, 1 320, 1 260, 1 200, 1 170, 1 100, 970, 890 and 845 cm^{-1}.

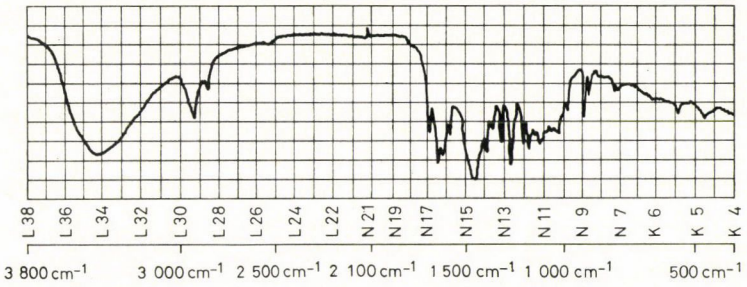

Fig. 2. IR spectrum of *Fusarium* toxin *F-2*

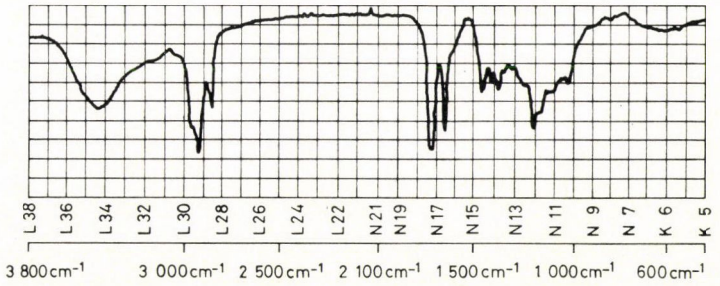

Fig. 3. IR spectrum of *Fusarium* toxin *F-3*

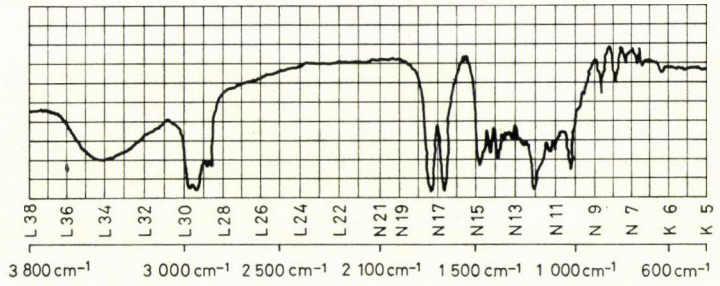

Fig. 4. IR spectrum of *Fusarium* toxin *F-4*

This is in fair agreement with the IR spectrum of zearalenone reported by other workers (MIROCHA *et al.*, 1968a.), and so are their chemical and biological properties. A typical IR spectrum is shown in Fig. 2.

The spectrum of the compound we named *F-3* slightly differed from that of zearalenone. Although the absorption maxima are similar in character at wave numbers 3 400 and 2 860 cm^{-1}, absorption is missing at wave number 1 670 cm^{-1}, typical of ketone and lactone groups, while a further difference is at wave numbers 700 to 900 where absorptions hinting at typical skeleton vibrations of the resorcyl acid aromatic ring, are missing. Relations are shown in Fig. 3.

Absorption spectra of compounds *F-3* and *F-4* are rather similar except that the former exhibits stripes typical of the aromatic ring, indicating presence of the basic skeleton of resorcyl acid lactone.

Some results connected with feeding tests are summarized in Table 1.

Table 1

Some results of feeding tests

Animal species	Toxin in organs								
	Liver			Stomach			Inner genitals		
	F-2	*F-3*	*F-4*	*F-2*	*F-3*	*F-4*	*F-2*	*F-3*	*F-4*
Albino rat	+	+	+	+	+	+	+	+	+
Rabbit	+	+	+	+	+	+	—	—	—
Hare	+	+	+	+	+	+	—	—	—
Pig	+	+	+	+	+	+	+	—	—
Deer	+	+	+	+	+	+	—	—	—

Subsequently, biological activity tests were made with the purified product of toxin *F-4*, since this could be demonstrated in the organism of several animal species.

Biological activity of the compound was tested on six-year-old virgin albino rats, administered intramuscularly, in 14-day test periods, 50 γ of toxin *F-4*, four times each.

3. Conclusions

Earlier detected toxin *F-2*, further, zearalenone derivatives *F-3* and *F-4* of unknown composition could be isolated from maize infected with *F. graminearum* and *F. culmorum*.

Feeding tests permitted to demonstrate biologically active compounds *F -2*, *F-4* occurring in gastric content, liver and on occasions, in testicles of fed animals. Toxin *F-4* was produced in purified form and its oestrogenic effect demonstrated in rat tests. Of the total of eight zearalenone type toxins, four were recognized to have similar structural properties, and to affect biologically the normal sexual cycle causing serious losses in animal husbandry.

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Acta Alimentaria, Vol. 4. (2), pp. 199—209 (1975)

IMPROVEMENT IN THE TEXTURAL QUALITIES OF IRRADIATED LEGUMES

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(Received July 31, 1974)

Gamma-irradiation of pulses reduces their cooking time in varying degrees, as measured by a texturemeter. Initial higher hydration rate on soaking and cooking, stabilizes on prolonged cooking and results in better and uniform texture of irradiated red gram. Better retention of some of the vitamins B, observed in irradiated and cooked red gram, is attributable to the reduction in cooking time.

Most legumes take comparatively long time for cooking. Practices such as soaking in water, cooking under pressure and use of chemical additives like sodium bicarbonate, sodium or ammonium carbonate or trisodium phosphate are, therefore, often resorted to for hastening cooking time (SUBBARAO *et al.*, 1964). These generally result in some losses of water-soluble vitamins or in protein damage (SUBBARAO & SUBRAHMANYAN, 1950). Recently, efforts are also being directed to obtain, by mutation breeding, selection and hybridization techniques, seed strains with better nutritive value and improved cooking qualities (SWAMINATHAN *et al.*, 1970).

It has been shown that radiation treatment reduces the rehydration time of partially dehydrated vegetables like carrot, cabbage, *etc.* and cooking time of potato, burdock and onions (TAKANO *et al.*, 1970). Cooking is known to cause certain physical and chemical changes, such as in appearance, flavour, palatability and digestibility; thereby the organoleptic attributes of the raw product are also altered. A precise measurement of the extent of softening of food associated with cooking is a pre-requisite for assessment of textural qualities in cooked foods.

Various instruments of diverse nature like the shear press (HARTMAN *et al.*, 1963), tenderometer (PROCTOR *et al.*, 1955) and texturemeter (FRIEDMAN *et al.*, 1963) are used for measuring the texture of foods (BOURNE, 1966). The *Instron Universal Testing Machine* is also employed for measuring diversified textural attributes of foods including softening (BOURNE *et al.*, 1966). Other reported methods, which depend upon the determination of dispersed solids in the cooking water in relation to the cooking time, or upon the use of the principle of alkali lability (BENHAM, 1942), are quite cumbersome.

In the present studies, the potential for use of gamma radiation processing to improve texture, hydration and cooking quality of pulses, particularly

red gram, has been examined. The textural changes in irradiated pulses in terms of softening is measured by a texturemeter, constructed in our laboratory. The effects of radiation and cooking on some of the vitamins B have also been studied.

1. Materials and methods

1.1. Irradiation

Dry legumes such as bengal gram *(Cicer arietinum)*, field bean *(Dolichos lablab)*, peas *(Pisum sativum)*, rajmah *(Phaseolus vulgaris)*, black gram *(Phaseolus mungo)*, lentil *(Lens esculenta)* and red gram *(Cajanus cajan)* were purchased from the local market. 100-g lots of the pulses were packed in polythene bags and exposed at ambient temperature (25 °C) to a ^{60}Co source of gamma radiation (*Gamma Cell-220*, ATOMIC ENERGY OF CANADA LTD.) having a flux of 15 krad min^{-1}, at dose levels from 0.5 to 3 Mrad. Absorption of radiation was checked with ferrous sulphate and ceric sulphate dosimetry (WEISS, 1952).

1.2. Construction of texturemeter

The sketch and photograph of the texturemeter constructed in this laboratory, are shown in Figs. 1 and 2, respectively. It consists of a stainless steel cylindrical chamber fitted with a removable perforated stainless steel disc at the bottom and resting on a tripod stand. A movable piston operates from the top. The test material is put between the disc and the piston to extrude it and pressure is applied on the piston by an arbor press. The free end of the lever of the arbor press carries a pan (Fig. 2). Weights are added to the pan till a complete removal of the cooked material through the perforated disc takes place. This is monitored by an electro-mechanical device actuating an indicator lamp, and recorded. The weights are added sufficiently quickly to avoid discontinuity in the extrusion of the test material.

Changes in the texture of the cooked pulse were also measured on the *Instron* (Table Model). This consists of a moving cross-head, which can be driven synchronously at a number of set speeds. A variety of attachments to squeeze, cut or puncture the test food, can be fitted to the cross-head and the force required is recorded on a strip chart recorder.

1.3. Determination of the rate of hydration

The pulse (5 g) was soaked for from 10 to 180 min in distilled water. In separate experiments, the pulse (5 g) was cooked for 2 to 25 min. At different time intervals, excess water was decanted, the samples wiped on a blotting paper and weighed. The rate of hydration was calculated by the difference in weights before and after soaking or cooking, as the case may be.

1.4. Estimation of vitamins B

Thiamine, riboflavin and niacin were estimated by methods described by A.O.A.C.(1960). Red gram flour was homogenized in 0.1 *N* HCl and autoclaved at 121 °C for 30 min. Thiamine and riboflavin were released by incubating the acid extract with takadiastase (1 mg ml^{-1}) at 37 °C for 20 h. Thiamine was eluted on activated *Decalso* column (10×0.5 cm). Oxidizing reagent

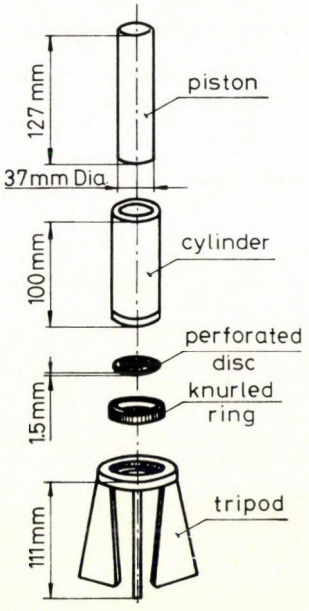

Fig. 1. Sketch of texturemeter. A stainless steel cylindrical chamber, fitted with a perforated disc (154 perforations, 52 thou) at bottom end and a movable piston operating from the top, is rested on a tripod stand. Pressure is applied by an arbor press to extrude the test material, placed between the disc and the piston

[1% $K_3Fe(CN)_6$ in 15% NaOH] was added to the eluate immediately followed by isobutyl alcohol. Tubes were shaken vigorously, centrifuged and solvent layer removed carefully. Thiochrome fluorescence was measured using quinine sulfate solution (0.25 μg ml^{-1}) to govern reproducibility of fluorometer. For riboflavin estimation, 1 ml glacial acetic acid (*Analar*) and 0.5 ml 4% $KMnO_4$ solution were added to 10 ml of the acid extract. After 1 min, fluorescence was measured before and after the addition of 20 mg of $Na_2S_2O_4$ and riboflavin content calculated. Niacin was determined colorimetrically using sulfanilic acid and cyanogen bromide reagents.

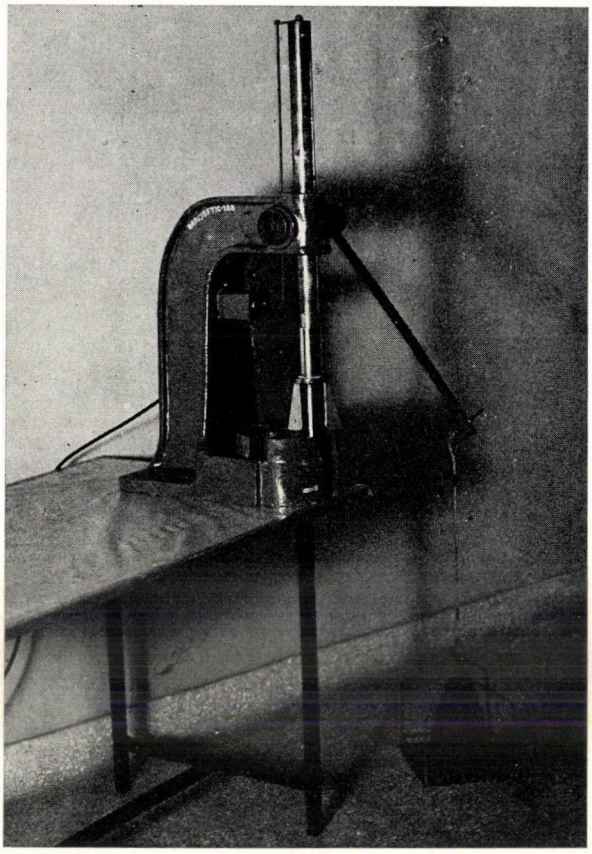

Fig. 2. The arbor press and the texturemeter. One end of the lever is attached to the texturemeter and the other to a pan. Red light monitors complete extrusion of the test material

2. Results

2.1. Effect of irradiation on the cooking time of the legumes

Reliability of the texturemeter, described in detail in "Materials and methods" to determine the extent of cooking, was demonstrated with several commonly consumed legumes. It can be seen (Table 1) that pulses required different times for complete cooking in terms of softening. The legumes were arbitrarily categorized into two groups, depending upon the time required for their cooking. Split pulses belong to the first category requiring less than 30 min and whole pulses to the second one which requires more than 30 min (Fig. 3).

Radiation (1 Mrad) processing of pulses resulted in reduction of cooking time varying from 8 to 39% (Table 1), red gram showing the maximum reduc-

Table 1

Cooking time of control and radiation processed pulses

Time required for complete cooking of pulses was determined as described in the text (para. 2.1.). The per cent reduction in cooking time, effected by radiation (1 Mrad), was calculated

Pulse	Cooking time (min)		Per cent reduction
	Control	Irradiated	
Split:			
Bengal gram	30	25	16.6
Red gram	26	16	38.5
Field bean	28	25	10.0
Black gram	22	20	9.0
Lentil	7	5	28.0
Whole dry:			
Peas	75	65	13.3
Bengal gram	60	55	8.3
Red Rajmah	55	45	18.2
White Rajmah	50	44	12.0
Lentil	40	35	12.5
Black gram	35	25	28.0

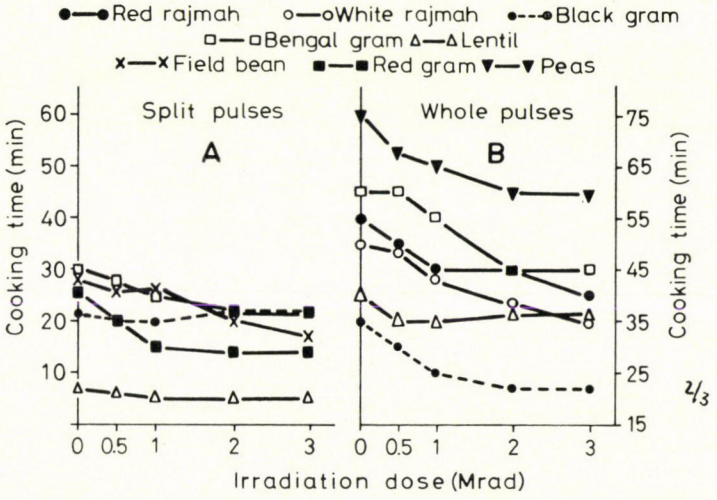

Fig. 3. Effect of irradiation on the cooking time of pulses. Pulses (in 5-g lot), irradiated at different dose levels, were cooked in 100 ml of boiling water and cooking time was determined as described in the text (para. 2.1.). Pulses requiring less or more than 30 min for cooking, are depicted in Figures A and B, respectively

tion. Though significant reduction in cooking time at higher doses (2 to 3 Mrad) was observed with most of the legumes (Fig. 3), excessive browning and off-odours on cooking made them unacceptable. Subsequent studies were, therefore, restricted to the red gram samples irradiated only with 1 Mrad.

A typical curve (Fig. 4A) obtained with red gram, unirradiated and irradiated (1 Mrad), shows exponential relationship of cooking time with the softening of the pulse. The pulse was taken as cooked when the weights added to the pan were almost constant. The weights added varied from 22 kg for the control pulse cooked for 2 min to 2 kg for that cooked for 26 min. It can be seen that irradiated samples took shorter time (16 min) than unirradiated ones (26 min). A straight line graph was obtained by plotting the values on a semilogarithmic paper (Fig. 4B). Texturemeter readings and their standard deviations are given in Table 2. It was interesting to note that, for each interval of cooking time, the values for standard deviations were much less for the irradiated pulse compared to those for the control.

Table 2

Texturemeter measurement of irradiated (1 Mrad) cooked red gram

5-g lots of red gram were cooked in boiling water (100 ml) for different periods and the extent of softening was measured in each case using the texturemeter. Results are mean values of 10 readings with standard deviations calculated

Cooking time (min)	Texturemeter readings		Standard deviation	
	Control	Irradiated (1 Mrad)	Control	Irradiated (1 Mrad)
4	15.76	9.04	1.83	0.16
6	13.67	6.06	2.05	1.04
8	10.24	4.88	2.22	0.16
10	8.12	3.40	1.26	0.15
12	7.70	2.44	1.51	0.12
14	6.14	1.44	0.20	0.02
16	4.58	0.90	1.10	0.01

Stress-strain curves obtained with *Instron* for irradiated (1 Mrad) red gram, cooked for 15 to 30 min, are shown in Fig. 5. The curve for unirradiated sample was not obtained even after cooking for 30 min, as the strain exceeded the stress. This was due to unavailability of suitable compression cells capable of producing higher stresses. The area under the curve was measured by planimeter and plotted against time. This is shown in Fig. 6. The shape of the curve closely resembled those obtained with texturemeter (Fig. 4).

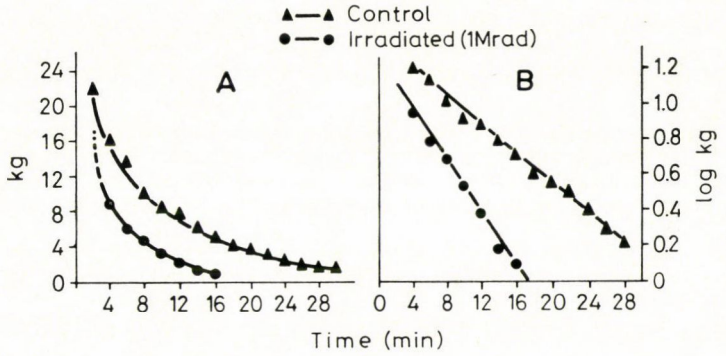

Fig. 4. Measurement of softening of cooked red gram. Red gram samples were cooked for different time intervals and the weight required for complete extrusion through the perforated disc of the texturemeter, was recorded. *A* shows the exponential and *B*, the logarithmic relationship of cooking time with the softening. Each point represents the mean value of ten independent determinations

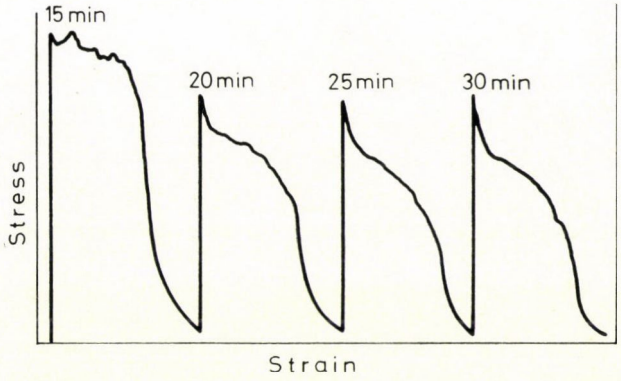

Fig. 5. Stress-strain curve of irradiated and cooked red gram. This was obtained in *Instron*, using compression cell (0 to 100 kg) at 100 full scale loading. The cross-head speed was 10 cm min^{-1} and the chart speed 100 cm min^{-1}

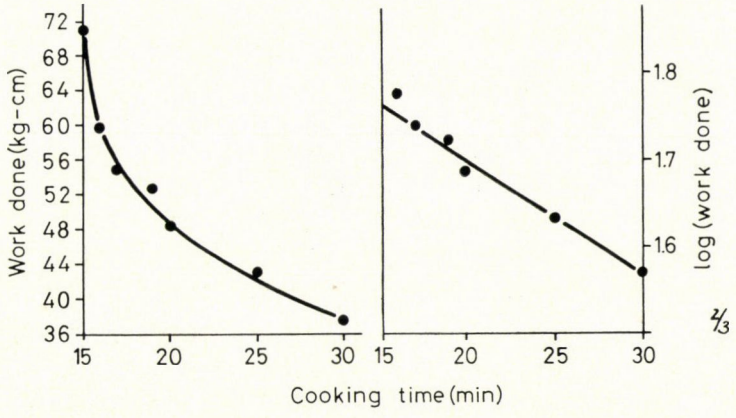

Fig. 6. Textural measurement of irradiated (1 Mrad) red gram. The work done was calculated from the area of the stress-strain curve (Fig. 5) and plotted against cooking time

2.2. Hydration properties of red gram

The rates of hydration of unirradiated and irradiated (1 Mrad) red gram samples on soaking (3 h) and on cooking (during 30 min) in water were compared. These are shown in Figs. 7A and 7B, respectively. Hydration rate of the irradiated sample was higher up to 1 h of soaking; no significant difference was observed thereafter. Water absorption capacity of the irradiated pulse was comparable during 5—10 min of cooking, after which this was about 10% less than in control samples. Prolonged cooking, for attaining desirable softness, resulted in rupture of unirradiated samples, while the irradiated ones remained intact.

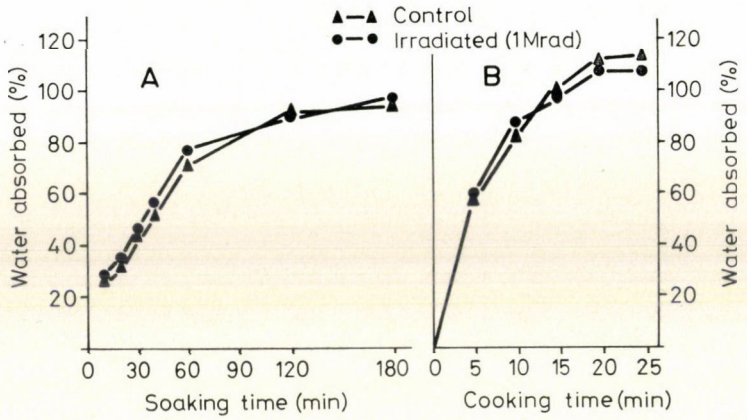

Fig. 7. Effect of irradiation on hydration rate of red gram. Red gram (5 g) samples were soaked in 100 ml water at 25—27 °C (*A*) or cooked in boiling water (*B*). At indicated times, samples were removed, blotted and weighed quickly

2.3. Effect of irradiation and cooking on vitamins B in red gram

Results on the effects of gamma irradiation and cooking on the retention of some of the water-soluble vitamins in red gram are given in Table 3. Irradiated and control samples were cooked, respectively, for 16 and 25 min, the time required to obtain desirable softening (Fig. 4). It can be seen that in the uncooked, irradiated (1 Mrad) sample, destruction of riboflavin was negligible, whereas thiamine and niacin showed about 7% loss. However, the vitamins were retained better in the samples irradiated and then cooked, compared to the corresponding control ones.

Table 3

Retention of vitamins B in red gram on cooking
Retention of vitamins was calculated on 100% dry basis in the control unirradiated sample which analysed to 6.2 riboflavin, 4.6 thiamine and 46 niacin $\mu g\ g^{-1}$

Treatment	% retention		
	Riboflavin	Thiamine	Niacin
Irradiated (1 Mrad, uncooked)	98.7	92.7	93.3
Control (cooked)	88.0	76.1	83.3
Irradiated (cooked)	95.0	82.7	89.3

3. Conclusions

The present investigation was undertaken to study the changes in textural qualities of pulses, with special reference to red gram, as affected by irradiation and cooking. The reliability of the texturemeter constructed in our laboratory was tested for the measurement of the extent of softening during cooking for several pulses. The close resemblance of the curves obtained with texturemeter (Fig. 4) and the *Instron* (Fig. 6) proves the sensitivity and efficacy of this appliance in measuring textural changes in spite of its simple design. It has been successfully used for determining the textural qualities of irradiated canned green peas (SHIRKHANDE, 1974). The texture of the irradiated sample, as judged by its shear-press values (kg per g pulse) at any given time, was softer than the unirradiated one. One of the desirable attributes in legume is short cooking time. To achieve the same degree of softness on cooking, irradiated red gram took about 40% less time than unirradiated samples (Table 1). BHATIA *et al.* (1967) have described a process, involving partial precooking with subsequent rehydration and proteolytic digestion, to reduce cooking time of red gram. This process also improves the solubility of red gram proteins (TARA *et al.*, 1972).

Another noteworthy point is the very low standard deviations in the texturemeter reading obtained with irradiated samples (Table 2). This reflects uniformity in the texture of the irradiated, cooked pulse samples, a desirable attribute in the development of a good quality product. The variation in the reduction of cooking time for different irradiated pulses (Table 1) indicates their variable response to radiation processing. This may be due to compositional and structural differences of the pulses. However, in general, the pulses with intact seed coats took comparatively longer time for cooking, and this may be due to their poor permeability to water (CAVAZZA, 1952).

The initial increase in the hydration rate of the irradiated red gram up to one hour of soaking and up to 5—10 min of cooking, indicates the constitutional changes in starch or proteins which facilitate hydration. It has been shown that the hydrophilic property of starch isolated from irradiated peas (KRYUK & MARKEVICH, 1962) and rice (TAKAOKA *et al.*, 1961) is altered. The decrease in the hydration rate of the irradiated pulse subjected to prolonged cooking correlates with the observed decrease in swelling power and increased solubility of the starch isolated from irradiated red gram (unpublished data). This is attributable to the depolymerisation of starch due to irradiation (WHISTLER & INGLE, 1967). Longer cooking for attaining desirable softness, resulted in rupture of the unirradiated samples due to bursting of starch granules, whereas the irradiated samples remained intact.

Radiation treatment, like most other conventional methods of food preservation, such as canning, dehydration and thermal treatments, could cause destruction of vitamins (RAICA *et al.*, 1972) to varying degrees depending upon their radiosensitivity, radiation dose, environment during irradiation and post-irradiation storage conditions (SRINIVAS *et al.*, 1974). Better retention of some of the vitamins B was observed in the samples irradiated and then cooked, compared to the corresponding control ones (Table 3). As prolonged heating is known to destroy vitamins B, the reduction in cooking time presumably accounts for the better retention of the vitamins in the radiation processed, cooked samples.

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A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Botyánszky Pál

A kézirat nyomdába érkezett: 1974. XI. 21. — Terjedelem: 9 (A/5) ív, 46 ábra

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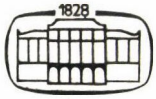

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Published by Morgan-Grampian Ltd., 30 Calderwood Street Woolwich, London SE 18 6QH England. Subscription rate: £10 ($30) per year.

P. Schmidt, E. Deutsch, J. Kriehuber

Diät für chronisch Nierenkranke

Eine Diätfibel für Ärzte, Diätassistenten und Patienten

2 Abb., 19 Tabellen
IX, 126 Seiten. 1973
DM 9,80; US $3.70
ISBN 3-540-06226-2
Kliniktaschenbuch

Zusammenstellung einer eiweiß- und elektrolytarmen Diät für chronisch Nierenkranke und für Dialysepatienten. Durch die übersichtlichen und abwechslungsreichen Tageskostpläne sowie die Einführung neuer Eiweiß- und Kalium-Einheiten außer für Ärzte und Diätassistenten auch für den interessierten Laien eine vorzügliche Fibel zum raschen Nachschlagen aller erforderlichen Angaben.

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18. V. 1975

ACTA ALIMENTARIA

ACADEMIAE SCIENTIARUM HUNGARICAE

VOL. 4 NUMBER 3

AKADÉMIAI KIADÓ, BUDAPEST

1975

ACTA ALIMENTARIA

ACADEMIAE SCIENTIARUM HUNGARICAE

A QUARTERLY OF THE COMMITTEE ON FOOD SCIENCE
OF THE HUNGARIAN ACADEMY OF SCIENCES

Edited by

K. VAS

Co-ordinating editor:

I. ORBÁNYI

Address of the Editorial Office:

Central Food Research Institute
H-1022 Budapest, Herman Ottó út 15. Hungary

Acta Alimentaria is a quarterly publishing original papers on food science in English. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Acta Alimentaria is published in quarterly issues comprising about 400 pages per year.

Subscription price: $ 32.00 per volume

Distributor:

KULTURA

Hungarian Trading Co. for Books and Newspapers
Budapest 62, P.O. Box 149, Hungary
or its representatives abroad, listed on p. 3 of the cover.

Acta Alimentaria is published by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
Budapest 502, P.O. Box 24, Hungary

Acta Alimentaria, Vol. 4 (3), pp. 211—216 (1975)

THE ZEATIN CONTENT OF TOMATOES AS A FUNCTION OF THE DEGREE OF RIPENESS, IRRADIATION AND STORAGE TIME

PRELIMINARY STUDY

E. KOVÁCS and ZS. VÖRÖS

(Received December 7, 1973)

The zeatin content of the *San Marzano* variety tomatoes was studied as a function of their degree of ripeness (fruitlet, green fruit, pink fruit, red fruit), storage time (at 20—23 °C and 40—50% RH) and radiation treatment (4 krad). The zeatin content was found to decrease as a function of the degree of ripeness.

The zeatin content of the fruitlet and of the green fruit was significantly ($P \geq 95\%$) higher than that of the ripe fruit. The other samples did not differ significantly.

The pink and red tomato fruits showed a slight loss of their zeatin content during storage. Determination was carried out on the 9th day of storage.

The zeatin content of pink tomatoes increased immediately upon irradiation with 4 krad. The difference was found to be significant (at the probability level of $P = 95\%$). The zeatin content of radiation treated and untreated tomatoes decreased during the 9-day storage time. On the 9th day the zeatin content of the irradiated tomatoes was 20% higher than that of the untreated ones.

The results of various experiments (MARTIN *et al.*, 1954; LETHAM, 1961; GERTMAN & FUCHS, 1972; THOMAS & ISENBERG, 1972; PIERIK & ABBADI, 1972) seem to prove the assumption that the ripening and aging of fruit is determined by the interplay of various hormone groups, such as auxins, gibberellins and cytokinins.

Probably cytokinins play a decisive role in the regulation and inhibition of aging.

The most active and the first to be isolated of these purine derivatives was zeatin (LETHAM, 1966; LETHAM & WILLIAMS, 1969).

The literature contains only a few studies which follow up the cytokinin concentration of fruits during their development, ripening and aging.

The aim of this study was to determine the zeatin content of some horticultural produce, radiation treated and untreated, during ripening and aging.

A more thorough knowledge of the biology of these horticultural products, the search for regularities enabling the researcher to establish the degree of ripeness in an objective way, was aimed at.

1. Materials and methods

1.1. Raw material

The *San Marzano* tomato variety was used in the experiments. The material was obtained from the Tordas Experimental Station of the NATIONAL INSTITUTE FOR VARIETY TESTING (Hungary).

Degree of ripeness:	Fruitlet (13 mm diameter), green stage, pink stage, red stage.
Date of picking:	Fruitlet, July 17; green: July 23; pink and red: July 30.

1.2. Storage

The pink and red tomatoes were stored at room temperature (20—30 °C, 40—50% RH).

1.3. Radiation treatment

Tomatoes in the pink stage of ripening were irradiated at the irradiation plant of the CENTRAL FOOD RESEARCH INSTITUTE (Budapest) in the radiation source of 60 kCi ^{60}Co activity. The radiation dose applied was 4 krad at a dose rate of 90 krad h^{-1}.

1.4. Extraction

The cytokinins were extracted by the method described by LETHAM and WILLIAMS (1969). The raw material was homogenized in ethyl alcohol three times the quantity of the sample. The homogenate was kept in the refrigerator overnight, then filtered. The filtrate was evaporated under vacuum (at 30 °C) and set at pH 3.0. The concentrate was shaken with a fourfold volume of ether. The ether phase, being cytokinin inactive, was not used. The aqueous phase was set at pH 6.5 and shaken with a fourfold volume of *n*-butanol saturated with water. The butanol phase was evaporated under vacuum (at 30 °C) and then applied to the thin-layer for chromatography.

1.5. Chromatography

A Kieselgel F_{254} layer (0.25 mm) was used for chromatography. The upper phase of an *n*-butanol – formic acid – water mixture of 10 : 4 : 5 was used to develop the chromatogram, according to LETHAM and WILLIAMS (1969).

The zeatin content was expressed as zeatin mg kg^{-1} raw material. 100 μl of a standard zeatin (CALBIOCHEM) solution (10 mg/25 ml 0.1 *N* NaOH) and 20 μl of the kinetin (CALBIOCHEM) solution (60 mg/25 ml 0.1 *N* NaOH) were applied to the thin-layer.

Of the samples 300, 500 and 900 μl, resp. were applied.

The chromatograms were photographed in UV light (254 nm) and the photos were evaluated in a *Chromoscan* Thin Layer Attachment (JOYCE & LOEBL) densitometer.

2. Results

2.1. Zeatin concentration as a function of the degree of ripeness

Zeatin was shown to be present in green *San Marzano* tomato samples (Fig. 1).

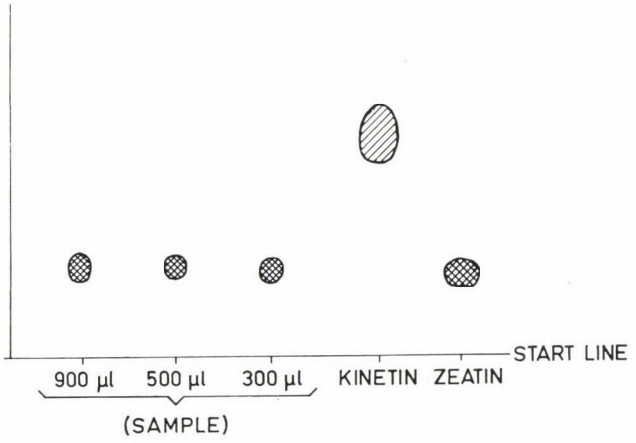

Fig. 1. Zeatin content of the butanol extract gained from green tomatoes, determined by thin-layer chromatography. Thin-layer: Kieselgel F_{254}. Solvent: *n*-butanol—formic acid—water (10 : 4 : 5, upper phase). Zeatin 110 µl, kinetin 20 µl samples 300, 500 and 900 µl, resp.

The zeatin content of tomatoes decreased with the advancement of ripening. The highest level was found in tomatoes in the initial stage of development and the lowest in red tomatoes (Fig. 2).

It is evident that

— the difference in the zeatin contents of the tomatoes in the initial stage of development and of red tomatoes was significant at the 95% probability level;

— the differences in the zeatin contents of the green and red tomatoes were also significant at the 95% probability level.

2.2. Zeatin content as a function of storage time

It was established in earlier experiments (KOVÁCS & VAS, 1969) that tomatoes in the pink or red stage of ripeness are suitable for storage. Therefore in these experiments we used tomatoes in these two stages of ripeness for the storage experiments. The samples were stored at room temperature [20—23 °C, 40—50% relative humidity (RH)] for 9 days. Storage was terminated when the pink tomatoes turned red and this happened on the 9th day.

The zeatin content of pink and red tomatoes as a function of storage time is illustrated in Fig. 3.

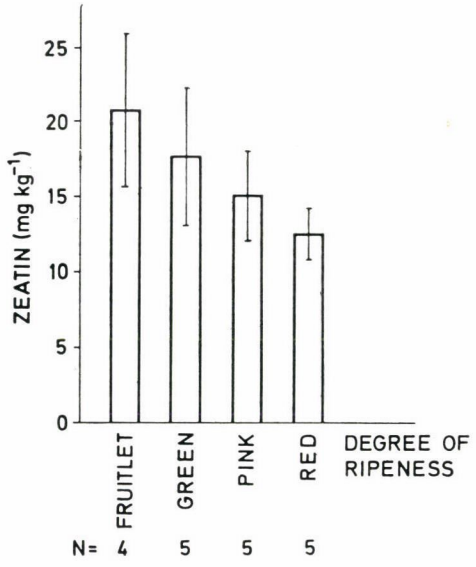

Fig. 2. Zeatin content of tomatoes as a function of ripeness. Vertical lines stand for standard deviation

	t values		
	green	pink	red
fruitlet	0.995	2.101	3.364**
green		1.148	2.501*
pink			1.566

* difference significant (P > 95%)
** difference highly significant (P ≥ 99%)

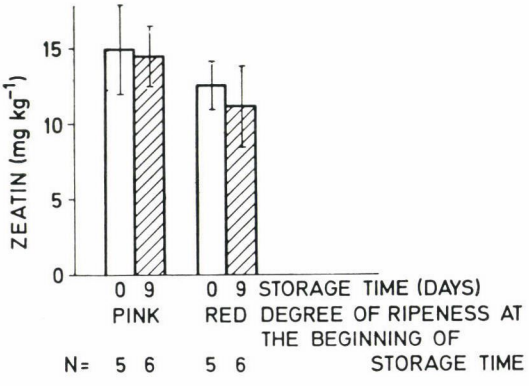

Fig. 3. Zeatin content of tomatoes as a function of storage time (at 20—23 °C and 40—50% RH). Vertical bars stand for the standard deviation

As seen, a slight decrease occurred in the zeatin content during storage. The difference, however, was not significant. Presumably after longer storage times the differences would have been greater.

2.3. The zeatin content as a function of radiation treatment

The storage life of pink and red tomatoes may be extended by irradiation (KOVÁCS & VAS, 1969).

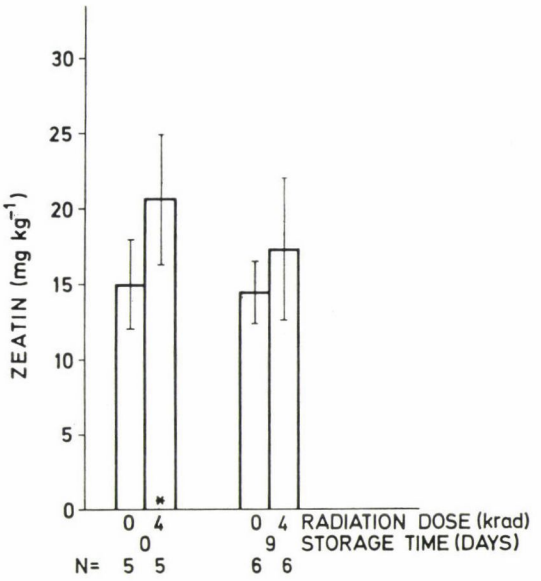

Fig. 4. Zeatin content of pink tomatoes as affected by radiation treatment. Treatment: radiation dose: 4 krad; dose rate: 90 krad · h^{-1}; storage conditions: 20—23 °C, 40—50% RH. * Differing significantly ($P \leq 0.05$) from the untreated sample

The extension of storage life achieved by treatment with low doses is due probably to changes in the activity of hormone systems. Similarly the growth of plants may be stimulated by irradiation with low doses.

On the basis of the above experiences, a 4 krad dose and tomatoes in the pink stage of ripeness were chosen.

The zeatin content of freshly picked pink tomatoes increases immediately upon treatment. The difference is significant at the 95% probability level (Fig. 4).

After 9 days storage the zeatin content of both irradiated and untreated tomato samples decreased. The extent of reduction was not significant. The zeatin content of the untreated sample was reduced by 5%, that of the irradiated tomatoes by about 15% during 9 days.

3. Conclusions

Cytokinins are mostly determined by biological tests. The result of quantitative evaluation is expressed in zeatin or kinetin equivalent.

In earlier studies we, too, applied biological tests (KOVÁCS, 1972). Experiments carried out then showed the cytokinin content of tomatoes to decrease with the advancement of ripening.

The results obtained in the present experiments by thin-layer chromatography support the earlier results.

The experiments carried out so far were of preliminary character. In further experiments we wish to identify zeatin by the use of different solvent mixtures in chromatographic analysis and to evaluate the chromatograms by biological tests.

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Acta Alimentaria, Vol. 4 (3), pp. 217—227 (1975)

DEGRADATION OF LEAF PROTEIN CONCENTRATES BY PEPSIN AND TRYPSIN

H. BOLDIZSÁR, P. RIBICZEY-SZABÓ and M. KOZMA

(Received December 21, 1973)

In vitro digestion tests were carried out with leaf protein concentrates prepared in Hungary. Extracts of Sudan grass, leafy sugarbeet-top and lucerne as well as *Hammarsten* casein, applied as a control, were subjected to pepsin and subsequently to trypsin digestion. The amino acid composition and the quantity of free amino acids, liberated by enzymes in the two-step degradation of the samples with a total N content equal to that of good-quality legumes, was determined. The protein N/non-protein N ratio in the leaf protein samples was found very favourable, while the essential/non-essential amino acid ratio was satisfactory. The efficacy of enzymatic degradation of the leaf protein samples was, in general, equal to that of casein as measured by the total amount of free amino acids and peptides liberated by pepsin and trypsin digestion. The amino acid composition of the concentrates was compared to the amino acid requirement of young pigs and the complementation of the concentrates with methionine was proposed. Several methods of informative character are known to study the digestibility of leaf protein concentrates. The Chemical Score, EAA and the "enzyme digestion index" of the individual products were determined by the methods described in this paper. In computing the index values casein was used as reference material. It was found that as compared to data given in the literature, the "enzymatic digestion index" may be considered satisfactory and is suitable to establish the biological value expected of the protein base.

The increasing protein deficiency encourages researchers to seek for new sources of protein. In recent years the interest in the so-called leaf proteins increased (STAHMANN, 1967; PIRIE, 1969). Efforts to produce leaf protein in Hungary have been strongly supported by the STATE OFFICE OF TECHNICAL DEVELOPMENT (OMFB) since 1965. Related literature has been largely reviewed in the latest publication of the Protein Program Bureau of the OMFB (KRALOVÁNSZKY-BÓCSA & BUGLOS, 1972).

The direct application in nutrition of the green parts of plants was hindered mainly by their high fibre and moisture contents and low protein content. However, appropriate extraction methods permit of producing protein concentrates free of fibres and moisture. In organisms of monogastric digestive system, the feeding of protein concentrates above a certain level is accompanied by disturbing moments: disagreeable odour and flavour and unfavourable digestibility and, with the lucerne, the saponin content. By the application of proper technology, these negative characteristics may be reduced to the minimum. On the side of the application of leaf proteins stands the fact that the amino acid composition of concentrates, known from the

literature, is adequate and in certain cases almost equivalent to animal protein. The biological value of the protein extracts gained from green plants depends, among many other factors, mainly on the enzymatic degradation in the digestive system, and is not known sufficiently yet.

The methods for the assessment of the biological value based on the amino acid composition of the protein, such as the Chemical Score, suggested by MITCHELL and BLOCK (1946) and the Essential Amino Acid Index, as suggested by OSER (1959) compare the values obtained upon acid hydrolysis with the amino acid composition of a reference protein. These methods do not take into consideration the processes taking place in the body, the digestion characteristics of the animal, the digestibility and susceptibility to enzymatic degradation of the feed, the losses upon preparation, the availability of lysine, *etc.* In order to establish the biological value of a feed, animal feeding trials are necessary. These methods are, however, rather time-consuming. Therefore, *in vitro* methods are to be found which are relatively rapid, yet take into account the most important biological factors, first of all enzymatic procedures, taking place in the digestive system.

In Hungary the production of leaf protein concentrates has been going on for several years, while biological studies connected with their application were initiated only lately. It seems advisable to extend research to the enzymatic digestibility and biological value of the concentrates.

In conformity with the above, the products of pepsin, pepsin-trypsin digestion and the amino acid composition of the hydrolysate of four Hungarian-made leaf protein concentrates were investigated.

1. Materials and methods

1.1. Materials

The four powdered leaf protein concentrates used in the experiments were as follows: Sudan grass extract, marked A-60-2; leafy sugarbeet-top extract, marked AM-20; two lucerne extracts marked A-50-2 and A-509-232. *Hammarsten* casein was used as reference protein. The samples were prepared as experimental products and put at the authors' disposal by the Protein Program Bureau of OMFB. The crude protein contents of the concentrates are given in Table 1.

1.2. Measurement of in vitro enzymatic digestibility and determination of amino acids

The total N content of the powdered samples was determined by *Kjeldahl*'s method. The amino acid composition was assessed after a 20-hour hydrolysis in 6 *N* HCl.

Enzymatic degradation was carried out in the following two steps:

400 mg powdered leaf protein was digested during 24 hours at 38 °C with 5 mg pepsin (SIGMA, 2 700 U mg^{-1}) in 50 ml HCl set at pH 1.8. The enzymatic degradation was interrupted in a 25-ml portion of the sample by placing it for 10 min in a boiling water bath. After centrifuging, the amino acids formed during the pepsin digestion were determined from the supernatant.

Another 25 ml of the sample were neutralized with 0.2 *N* NaOH. The pH value was adjusted to 7.6 with 0.1 *N* Na_2HPO_4—KH_2PO_4 buffer solution. The sample was made up to 50 ml and for further digestion 10 mg trypsin were added (MERCK, 2.0 U mg^{-1}) and kept at 38 °C for 24 hours. During digestion with pepsin and trypsin toluol was added to the samples to prevent microbial activity. Finally the sample was brought to a boil and kept for 10 min in a boiling water bath to denature protein. The samples were then centrifuged and the supernatant tested for residual protein with trichloro-acetic acid. The amino acids were determined in the supernatant.

In order to assess the peptide content formed during enzymatic degradation an aliquot of the supernatant was hydrolyzed with hydrochloric acid and the amino acids thus obtained were determined.

For the sake of comparison the total N content of the supernatant was also determined.

The amino acids were analyzed with *Biochrom* automatic amino acid analyzer. The two-column technique was used and an amount corresponding to an expected value of 0.5 μmole was applied to the column. Taurin was used as internal standard. With threonine, serine, cystine and tyrosine, a correction was applied to account for degradation.

The tables contain the amino acids in the sequence of their elution. The values for glutamine and glutamic acid on one hand and asparagine and aspartic acid on the other are given jointly.

2. Results

The amino acid composition of the leaf protein concentrates tested, of their pepsin and pepsin—trypsin hydrolysates and the amount of amino acids as determined in the hydrochloric acid hydrolysate of the supernatant are shown in Table 1. The same table contains the total N values for the concentrates and for the supernatant of enzymatic degradation. In the first two columns the analytical data of casein are given, first the official data determined by the National Research Council & National Academy of Sciences (COMMITTEE ON ANIMAL NUTRITION, 1968) and subsequently those obtained in the present study.

Table 1

Amino acid composition of leaf protein

(mg amino aci

Protein preparation tested / Amino acid	Casein *(Hammarsten)*					Sudan grass A-60-2			
	protein		pepsin	pepsin + trypsin	pepsin + trypsin*	sample*	pepsin	pepsin + trypsin	pepsin + trypsin*
	as stated by NRC[e]	as determined in this lab.							
Aspartic acid	6.6	7.8	0.08	0.09	3.64	3.69	0.14	0.24	1.09
Threonine	4.2	5.5	0.03	0.15	2.12	1.65	0.12	0.05	0.28
Serine	6.4	6.1	—	0.11	3.27	1.70	0.01	0.05	0.31
Proline	12.7	10.9	—	—	4.90	1.28	0.12	0.29	0.36
Glutamic acid	23.7	18.5	0.03	0.17	3.40	5.75	0.92	0.12	0.78
Glycine	1.7	1.6	0.04	0.13	0.93	1.84	0.08	0.57	0.59
Alanine	3.2	3.1	0.02	—	1.34	2.04	0.37	0.68	0.59
Valine	7.5	7.1	0.12	0.20	2.84	1.76	0.10	0.71	0.50
Cystine	0.3	0.03	—	—	0.19	0.27	—	0.76	0.50
Methionine	3.0	1.8	—	0.58	1.09	0.23	0.08	0.24	—
Iso-leucine	6.3	3.8	—	0.45	3.26	2.80	0.10	0.71	2.81
Leucine	9.5	3.3	0.36	0.45	3.26	2.59	0.33	0.54	0.75
Tyrosine	5.2	5.6	0.16	0.49	2.47	1.39	0.08	0.77	1.50
Phenylalanine	5.1	5.2	0.23	0.57	2.00	1.80	0.08	0.76	1.88
Lysine	7.7	6.3	0.06	3.15	4.90	3.00	0.14	0.09	0.71
Histidine	2.8	2.5	0 23	0.21	1.43	0.65	0.01	—	0.15
Arginine	3.8	3.5	—	0.27	1.47	2.17	0.01	—	0.12
Total	109.7	92.6	1.36	7.02	42.5	34.61	2.68	6.48	12.92
Crude protein (N · 6.25)		87.5			—	35.8			16.96

* Values obtained by hydrolysis with 6 *N* HCl

2.1. Protein and amino acid contents of the samples

The total N and the crude protein (N · 6.25) content were on an equal level to that of legumes of good quality, in the Sudan grass sample A-60-2: 5.72 g N per 100 g solids, in the lucerne sample A-509-232: 6.46 g N per 100 g solids and in the leafy sugarbeet-top marked AM-20: 4.58 g N per 100 g solids.

While the protein value of the conventional protein sources is adequately characterized by the total N content, a substantial proportion (sometimes 50 %) of the total N content of leaf proteins, as reported in the literature, is non-protein N. ARCKOLL (1971), ALLISON and CHUA (1970) have demonstrated this in grasses, ALLISON and CHUA (1970) for lucerne. In view of the above the amino acid composition of the leaf protein extracts after hydrolysis was also

concentrates and their enzymatic hydrolysates

per 100 mg solids)

Lucerne A-50-2				Leafy beet-top AM-20				Lucerne A-509-232			
sample*	pepsin	pepsin + trypsin	pepsin + trypsin*	sample*	pepsin	pepsin + trypsin	pepsin + trypsin*	sample*	pepsin	pepsin + trypsin	pepsin + trypsin*
3.72	0.20	0.38	1.69	1.98	0.17	0.54	1.19	0.55	0.30	0.23	1.22
1.65	0.14	0.23	0.69	0.92	0.56	0.09	1.12	2.07	0.51	0.10	0.51
1.51	0.69	0.13	0.59	0.94	0.15	0.04	0.59	2.04	—	0.07	0.44
1.34	0.06	0.15	0.48	0.69	0.02	0.05	0.31	2.50	0.21	0.15	0.53
5.77	1.41	0.88	2.07	3.18	—	0.31	1.59	4.71	0.45	0.40	1.31
1.65	0.04	0.38	0.61	0.95	0.04	0.09	0.68	1.49	0.05	0.12	0.47
1.85	0.35	0.48	0.87	1.33	0.37	0.21	0.53	2.57	0.31	0.27	0.53
3.94	0.09	0.44	0.98	1.11	0.05	0.29	0.68	2.35	0.10	0.36	0.49
0.29	—	0.04	0.03	0.25	—	0.03	—	0.30	0.02	0.18	—
0.28	0.07	0.34	0.12	0.30	0.07	0.18	—	0.85	0.01	0.08	—
3.20	0.10	0.54	1.43	0.01	0.04	0.35	0.78	1.85	0.12	0.36	0.59
0.95	0.001	1.01	1.71	0.75	0.11	0.62	0.56	3.51	0.29	0.71	0.42
0.48	0.09	0.50	0.28	0.51	0.12	0.48	0.41	2.68	0.13	0.45	0.39
1.20	0.07	0.45	0.40	0.75	0.08	0.43	0.53	2.42	0.19	0.45	0.48
2.15	0.06	0.36	0.57	1.72	0.02	0.57	0.89	2.75	0.03	0.97	1.03
0.35	0.15	—	0.06	0.55	0.01	0.06	0.04	0.80	—	—	0.25
1.35	—	—	0.17	1.13	0.28	—	0.34	0.60	—	—	0.55
31.68	3.52	6.31	12.75	18.07	2.09	4.34	10.24	34.04	2.72	4.90	9.21
35.7			10.4	28.6			12.6	40.4			12.6

Enzymatic hydrolysis: with pepsin (SIGMA = 2 700 U mg^{-1}; pH = 1.8; 24 hours; t = = 38 °); with pepsin and trypsin (MERCK = 2.0 U mg^{-1}; pH = 7.6; t = 38 °C; 24 hours). Amino acid analysis: *Biochrom* automatic analyzer, applied to columns Aminex-A5 and Aminex-A6. Crude protein determined by *Kjeldahl*'s method (N · 6.25).

determined. In the Hungarian samples the protein/non-protein ratio was very favourable. The total amino acid content determined formed 96.7, 88.7, 63.2 and 84.2 % of the crude protein content of Sudan grass, the 2 lucerne extracts and the leafy sugarbeet-top, respectively. In some cases the amino-N values as determined in the experiments and shown in Tables 1 and 2 are by about 10—12 % higher than the true values. This phenomenon may be explained by the fact that the amino acids, depending on their molecular weight, absorb water in amounts corresponding to about 9.0—19.3 % of their molecular weight. The total amino acid of casein as determined after hydrolysis amounts to 92.6 g

per 100 g solids. After subtracting the water absorbed during hydrolysis the amino-N value of 82.8% is obtained.

The essential/non-essential amino acid ratio in the extracts was satisfactory. In the Sudan grass the essential amino acid content constituted 48.1%, in the lucerne sample A-50-2 46.6%, in the A-509-232 lucerne 50.5% and in the leafy beet-top 45.5% of the total amino acid content. (The tryptophane content was not taken into account.)

2.2. Enzymatic degradation

The degradation efficiency of pepsin (considering the sum of the amino acids as 100%) was rather low, between 7.74 and 11.6%. In the samples treated by trypsin subsequent to degradation by pepsin, the free amino acids formed 14.4—24.1% of the total amino acid content (Table 2).

Table 2

Free amino acid content of leaf protein concentrates expressed as percentage of the amino acid content of the concentrate

Sample tested	Total amino acid content*	Hydrolysates obtained with		
		pepsin	pepsin + trypsin	pepsin + trypsin*
Casein	100	1.47	7.58	45.90
Sudan grass A-60-2	100	7.74	18.71	37.30
Lucerne A-50-2	100	11.10	19.90	40.20
Leafy beet-top AM-20	100	11.60	24.10	56.70
Lucerne A-509-232	100	7.99	14.40	26.81

* Value obtained by hydrolysis with 6 *N* HCl

The free amino acid content obtained upon enzymatic degradation was low, but in the chromatograms some unidentifiable peaks appeared. Without peptidases the enzymatic degradation of proteins is not complete, therefore it is obvious that a part of the amino-N is present in the supernatant or in the digest as peptide. By the acidic hydrolysis of the supernatant a further amount of amino acids was liberated and the values thus obtained were between 26.8% and 56.7%. The supernatant contained also non-amino-N.

2.3. Amino acid composition of the concentrates in view of the requirements of young pigs

The amino acid composition of the concentrates, as found upon hydrochloric acid hydrolysis, was compared to the requirements of the young pig

[for pigs of 20—35 kg body weight, in case of a diet containing 14% protein and 3 300 kcal kg^{-1} metabolizable energy, as recommended by the COMMITTEE ON ANIMAL NUTRITION (1968)]. Data are shown in Table 3.

Table 3

Comparison of the essential amino acid requirement of young pig with the amino acid content of leaf protein concentrates

Amino acid	Requirement	Sudan grass A-60-2		Lucerne A-50-2		Leafy beet-top AM-20		Lucerne A-509-232	
	amino acid % in the protein	amino acid % in the protein	content / requirement, %	amino acid % in the protein	content / requirement, %	amino acid % in the protein	content / requirement, %	amino acid % in the protein	content / requirement, %
Threonine	3.2	4.8	150	3.2	160	5.1	160	6.3	200
Valine	3.6	5.1	140	12.3	240	6.1	170	6.9	180
Methionine	3.6	0.7	20	0.9	25	1.7	47	2.6	72
Iso-leucine	3.6	8.1	220	10.1	280	5.6	150	5.6	150
Leucine	4.3	7.5	170	3.0	70	4.2	98	10.6	250
Phenylalanine	3.6	5.2	150	3.8	110	4.1	110	7.3	200
Lysine	5.0	8.7	170	6.8	130	9.5	190	8.3	170
Histidine	1.3	1.9	150	1.1	80	3.0	230	2.4	180
Arginine	1.4	6.3	450	4.3	300	6.3	450	1.8	130

As seen, the concentrates need to be complemented with methionine. The necessary complementation in the case of Sudan grass amounts to 1%, for lucerne A-50-2 0.8%, for leafy beet-top and lucerne A-509 0.35%. When thus supplemented, the Sudan grass and the lucerne A-509 extracts meet to 150% the essential amino acid requirement of the young pig. Two further amino acid components of the leafy beet-top, leucine and phenylalanine, are available in adequate amount. In lucerne A-50-2 beside methionine, leucine and histidine are of limiting character.

2.4. Biological value of leaf protein extracts

Several *in vitro* methods are known for the evaluation of the biological value of nutrients. The Chemical Score as calculated on the basis of the essential amino acid composition of the sample tested and the Essential Amino Acid Index according to OSER (1959) show some correlation with the biological value as established in rat experiments, however, the first method produces an unfavourable result, while the second one overestimates the protein. Only that part of the potential amino acid composition of the nutrient is utilizable which is liberated by the proteolytic enzymes of the digestive tract. The amino

acids formed upon digestion and those present in peptide chains are taken into account in the PPD index as recommended by AKESON and STAHMANN (1964). It was proven in many experiments by the authors that the relative digestibility value of the sample to be tested, related to a reference protein, that is the "calculated biological value" correlates closely with the biological value as measured in animal tests. A similar value, the "enzymatic digestion index", was computed from the analytical data of the samples. Although the methodology applied in this study differed somewhat from the analytical method of AKESON and STAHMANN (1964) in that a longer digestion period and instead of white of egg casein was used as the standard, the train of thoughts followed and the corresponding mathematical model was similar to the internationally accepted PPD index. Table 4 gives the values calculated from the amino acid composition of the leaf protein concentrates tested.

Table 4

Evaluation of leaf concentrates based on the Chemical Score, Oser's EAA Index and the "enzymatic digestion index"

Protein preparation tested	Protein extract		Enzymatic digest
	Chemical score	*Oser*'s EAA index	Enzymatic digestion index
Casein	54	91.3	79.0
Sudan grass A-60-2	20	83.6	55.7
Lucerne A-50-2	25	80.4	70.5
Leafy beet-top AM-20	47	91.7	65.1
Lucerne A-509-232	72	96.4	65.1

The Chemical Score values are low, due to the lack in the first limiting amino acid, methionine. However, it was reported in the literature (MITCHELL, 1962) that the complete deficiency of one essential amino acid does not render the protein of the nutrient unutilizable in tissue metabolism. Deficiency in an amino acid may be replaced by an amount originating from tissue catabolism. The Essential Amino Acid Index proved to be rather high in the case of the leaf proteins tested. The "enzymatic digestion index" of the Hungarian protein concentrates, using casein as reference protein, was found to be between 55.7 and 70.5. Taking into account the biological value of casein known from the literature, the relative protein indices may be considered satisfactory. Thus it may be that, complemented as needed, the leaf protein concentrate s, in accordance with the expectations, will be suitable to improve the protein basis.

3. Conclusions

The protein content of the examined concentrates produced in Hungary was satisfactory. Unless their use was not hindered by other circumstances, they might supply an important part of the protein required in feeding pigs.

A considerable proportion of the total-N present in the samples is formed by amino-N.

GERLOFF and co-workers (1965) have shown that the essential amino acid content of protein concentrates reaches the corresponding values of the corn variety Opaque-2 and of soy-beans. BYERS (1971) compared the essential amino acid content of 21 plant species with the human amino acid requirement as laid down by FAO and found them more favourable than the reference values of FAO. Leaf proteins are particularly rich in lysine. Various literature sources agree that the protein extracts gained from different plant species contain amino acids in very similar composition (PIRIE, 1969). Thus attention should be drawn to the fact that Hungarian-made concentrates have, in relation to traditional fodder-crops, a high threonine content and therefore are suitable for the complementation of other plant proteins.

Leaf protein concentrates are utilized mainly in pig breeding, therefore their amino acid content was compared to the amino acid requirement of pigs. In comparison to the 14—16% protein requirement of young pig the essential amino acid content of the 20—40% crude protein content of the concentrates significantly exceeds, in an absolute sense, the amino acid requirement of the animal species. Though at higher protein content the essential amino acid requirement relatively decreases (BRESSANI & MERTZ, 1958), the protein content of the feed will be covered only partly by the extracts, and therefore the amino acid ratios have to comply with the requirements of the animal species. It appears from Table 3 that the aminogramme of the extracts comes up to expectations: if supplemented by methionine the essential amino acids generally cover the requirement. It is worth considering, whether — as it was pointed out earlier (BOLDIZSÁR *et al.*, 1971) — supplementation with the second limiting amino acid would not be profitable. In that case complementing with arginine,* leucine or histidine would be required, taking into account at the same time the amino acid content of the other components of the feed, the animal species to be fed, its age, *etc.*

The idea of determining the biological value of proteins after enzymatic digestion emerged about twenty years ago. As a result of enzymatic hydrolysis a significant proportion of amino-N is regained in the form of peptides and the amount of free amino acid depends on the enzyme used for digestion.

* In view of the well-known arginine-lysine antagonism (D'MELLO & LEWIS, 1970), the important surplus of lysine in the concentrates increases the necessity of arginine complementation.

In the present study the aromatic amino acid content of casein was found to be about 11.6%, the lysine content 6.8%. After digestion with pepsin about 28.7% of the total free amino acid content consisted of tyrosine and phenylalanine, while only 4.4% of lysine. After digestion with trypsin the proportion of the two aromatic amino acids decreased to 15.1%, while the proportion of lysine jumped to 44.9%. The results are characteristic of the digestive specificity of pepsin and trypsin.

Pepsin-pancreatin digestion was applied by AKESON and STAHMANN (1964). The pancreas extract contained probably exopeptidases, possibly kathepsins as well. The authors consider the use of the more exactly definable trypsin in model experiments more suitable. AKESON and STAHMANN (1964) compared the amino acids obtained by enzymatic digestion of the plant proteins tested to the free amino acids gained by the enzymatic digestion of a reference protein (whole egg-white), considering thereby the fact that the sum of the amino acids released during the treatment is limited by the substrate specificity of the enzyme used.

The degradation efficiency data as obtained in this study seem lower than those given by AKESON and STAHMANN (1964). Apart from the difference in the enzymes used, this might be due to the differing enzyme substrate ratio (1.5 mg pepsin and 5 mg trypsin to 100 mg protein). A substantial part of the amino-N in the digest supernatant is present in the form of peptide evidenced by the non-identifiable peaks found in the chromatograms. Thus the total amino acid content of the supernatant was considered the final result of enzymatic action. After hydrolysis with hydrochloric acid 26.8—56.7% of the protein was regained. This result related to casein approximates the values achieved in an earlier study (HORVÁTH & BOLDIZSÁR, 1973) with CALBIOCHEM pronase of high proteolytic activity. The degradation efficiency of the natural digestive enzymes was lower with leaf proteins than with casein (Table 2), except with the leafy beet-top. However, the absolute value of the results seems satisfactory.

The knowledge of the amino acid composition of the protein source used in animal feed is not sufficient to the unambiguous assessment of the biological value. This value may be different for samples the amino acid composition of which is identical or similar. Primarily the difference is caused by the differing proportion of individual amino acids upon digestion by various enzymes. The study of enzymatic digestion under standard conditions enables the evaluation of enzymatic digestibility.

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The authors wish to express their gratitude to the members of the Protein Program Bureau of OMFB for making available the samples to be tested.

Thanks are due to Mrs. I. SEBESTYÉN-SZABADI for her devoted technical assistance.

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Acta Alimentaria, Vol. 4 (3), pp. 229—250 (1975)

INVESTIGATION OF STRING BEAN VARIETIES FOR THEIR SUITABILITY TO QUICK-FREEZING

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(Received January 18, 1974)

To establish their suitability for processing, green and yellow varieties of string bean were subjected to investigation. Nine varieties were studied of which three were put to storage tests.

The colour and texture, as well as in some of the samples the vitamin C content of fresh and quick-frozen or stored beans were studied. The samples underwent sensory evaluation and the changes during various treatments were followed up.

The colour tests proved that the colour of beans with green pods became more saturated upon quick-freezing and did not deteriorate substantially during storage at —20 or —30 °C. However, during storage at —10 °C it soon turned brown (Tables 1—4). The colour of yellow-pod beans deteriorated upon cooking and quick-freezing and turned brownish. The colour change was always significant.

Finometer was used to check the texture. The texture developed upon quick-freezing was observed to remain stable during storage. The changes caused by the various treatments are shown in histograms (Figs. 1—3), and by the regression correlation as seen in Fig. 4. With the bean varieties tested, the optimum cooking time for beans of about 70 *Finometer* degrees was found to be 15 min.

The effect of quick-freezing was examined also by the water-binding capacity of the samples. As seen in Table 5 the water-binding capacity hardly changed during 6 months of frozen storage from that registered directly upon quick-freezing. These results support those established by *Finometer* readings, namely that the texture developed upon quick-freezing does not change substantially during storage under appropriate conditions.

The vitamin C content of three varieties was tested during storage by thin-layer chromatography. During 10 months of storage at —10 °C the vitamin C content deteriorated completely. At —20 °C about 5% and at —30 °C about 10% of the initial amount were retained. Results are shown in Table 6.

The effect of quick-freezing (Table 7) and the keeping quality of certain varieties were evaluated by sensory testing, taking into account 30% or 50% deterioration of taste. Spoilage rate curves were plotted (Figs. 8 and 9) and the keeping quality at given temperatures was established (Table 9).

On ranking the varieties according to keeping quality *Sanocrop* was found to be the first, *Budai zöld* the second and *Harvester (green)* the third. However, ranking according to taste scores obtained directly upon quick-freezing showed the following sequence: *Sanocrop, Harvester (green), Budai zöld. Budai zöld* received the lowest score in the raw stage, therefore it is not suggested for quick-freezing, even if the taste deterioration during storage did not seem to exceed that of *Harvester (green)* and *Sanocrop*. Considering the changes in sensory value, in texture and in colour as caused by quick-freezing, of the green-pod varieties tested, *Carobel Wax* and *Jutta* proved to be the most suitable for storage after quick-freezing. The yellow-pod varieties having a *Finometer* value about 50—70 seemed all suitable for processing in the canning industry. However, the change in their colour upon quick-freezing does not permit of their use in the refrigeration industry.

In order to improve the quality of the products of the food industries, string bean varieties were exposed to comparative tests.

The problems most discussed were related to the colour and texture (BEKE, 1970; BURKA, 1970). Many authors investigated texture. Instrumental studies were reported on by REEVE (1970), BOURNE (1972), BOGNÁR (1972), *etc.* GUTSCHMIDT (1969) described the structural changes observed upon various freezing technologies in the water-binding capacity. Structural changes were followed by ZACKEL (1973) in histological studies.

As regards the colour of string beans WESCOTT and his co-workers (1955) investigated the non-enzymic browning, and VAN BUREN and his co-workers (1964) the change in chlorophyll as caused by precooking.

The subject of the present study was to establish the suitability of some green and yellow string bean varieties for quick-freezing and thereby select varieties suitable for processing in the food industry. In storage experiments the keeping quality of the varieties was studied.

1. Materials and methods

1.1. Materials

Green- and yellow-pod bean varieties were obtained from sources of the canning and refrigeration industries. Two green string bean varieties were grown for the COLD STORE, SZÉKESFEHÉRVÁR, and were obtained in the plant on delivery. Another variety was grown for experimental purposes in the SASAD COOPERATIVE. Five varieties were grown in the SOROKSÁR COOPERATIVE, partly for the CANNING FACTORY, NAGYKŐRÖS. One variety was bought from a private farmer on the day of picking.

By using different sources of cultivation and several varieties we aimed at giving a more general view.

The varieties used in the experiment in the sequence of picking dates are as follows:

Budai zöld (green)	9. 8. 1972	*Carobel Wax (green)*	13. 7. 1973
Harvester (green)	10. 8. 1972	*Jutta (green)*	13. 7. 1973
Sanocrop (green)	10. 8. 1972	*Harvester (yellow)*	17. 9. 1973
Kinghorn Wax (yellow)	13. 7. 1973	*Juliska (yellow)*	27. 9. 1973
Wax Goldjuwel (yellow)	13. 7. 1973		

The *Kinghorn Wax* variety was mechanically harvested, all the other varieties were hand-picked. Tests were started on the day of picking and the samples were kept in a storage room of +8 °C till finished.

Three varieties, selected on the basis of preliminary trials, were subjected to storage tests in 1972. The samples obtained in 1973 were not stored, they were tested in the raw, precooked state and upon quick-freezing.

1.2. Preparation and freezing of the test samples

Samples used in the storage tests were topped and tailed, cut into pieces of 2—3 cm, precooked in water of 95 °C for 2 min, cooled in tap water and packaged in cardboard boxes, each containing 0.4 kg. The samples were then frozen at —30 °C during 48 hours.

1.3. Storage temperatures

The frozen samples were stored at the following temperatures: -10 ± 4 °C, -20 ± 1 °C and -30 ± 1 °C, resp.

1.4. Test methods

The samples were tested in order to be able to evaluate each variety. The length, colour, texture and the vitamin C content of the pods were measured and the varieties were ranked according to their sensory value. Their keeping time was established by following up the quality during storage.

1.4.1. Colour tests. The colour was determined with the *Lovibond Tintometer*, Type AF 751. The *Lovibond* units thus obtained were then converted into C.I.E. units. On the basis of results the trichromatic numbers, the characteristic wavelength and the lightness factor may be obtained.

Samples were tested in the fresh, precooked, cooked, quick-frozen state and after frozen storage.

1.4.2. Finometer tests. The texture of the pods was measured with the *Finometer*, found very useful for testing the texture of peas (VÁGNER & CSENDES, 1968). The spring constant of the *Finometer* was $C = 13.25$ kg mm^{-1}. Pod slices of 2—3 cm length are easy to fit in the cup of the *Finometer* and it is easy to measure them.

Consistency was measured in the fresh, precooked, cooked and quick-frozen samples, directly upon freezing and after storage.

1.4.3. Texture test by measurement of the water-binding capacity. The water-binding capacity was measured by the somewhat modified variant of the GUTSCHMIDT (1969) method. This characteristic, namely juice release under a predetermined load, is also characteristic of the texture. The processing technology, the storage temperature and the time of storage manifest themselves in changes of the water-binding capacity. Thus this method was used to characterize textural changes in the pods.

The difference between the original *Gutschmidt* method and the modification lies in using a 2-kg load instead of a 3-kg one. The process is as follows: a piece of bean pod of about 1 g is accurately measured and placed between accurately measured filter papers and pressed for 2 min with a weight of 2 kg. Schleicher-Schüll 2040 filter paper was used for the test. After 2 min the sample pieces were again measured. The difference between the two measure-

ments was equivalent to the loss of juice. Results were expressed in percentage of the original weight.

This test was carried out with every bean variety after precooking for 2 min, cooking for 15 min and after quick-freezing at —30 °C. With the 3 samples subjected to storage test the measurement was repeated after 6 months storage at —10°, —20° and —30°C. The temperature of the samples was set at 20 °C for the measurements.

1.4.4. Determination of the vitamin C content. PETRÓ-TURZA's method (1969) was used with some modification. It was observed that string beans contained compounds (probably sugars) which appear at the Rf value characteristic of the vitamin C DAS-osazone formed in 2,4-dinitrophenyl hydrazine solvent and therefore cover the same. By varying the concentration of the pyridine used as running solvent a concentration was found at which the Rf value of vitamin C of the string beans was clearly different from that of other osazones.

The separation and determination by thin-layer chromatography of the vitamin C content of string beans was carried out by the following procedure:

50 g of string beans were comminuted in a *Turmix* blendor for 2 min with a 1 : 1 mixture of ethyl alcohol and metaphosphoric acid and filtered. Five-ml samples were taken to produce osazones of vitamin C according to the method used. The DAS-osazone thus formed was applied to the thin-layer in an ethyl acetate solution. Kieselgel-G was used for the layer and a benzene—acetone—pyridine mixture (80 : 16 : 4) as the developing solvent. The developed chromatogram, to make it more photogenic, was placed in the vapour space of a chromatography tank. To evaluate the chromatogram a known amount of a standard ascorbic acid solution was applied and visually compared (BLAZOVICH *et al.*, 1969).

1.5. Sensory evaluation

Sensory tests were carried out according to *Plank*'s system (SPANYÁR, 1954). The individual properties were scored on a 5-point scale, 5 points standing for excellent, 1 point for very poor quality. In order to achieve uniform scoring the panel members were given a list of criteria for every property. The panel consisted of 5 members.

The samples were scored for colour, odour, flavour and texture. A 30% loss of flavour score was considered the upper threshold value of a sensorically evaluable change of quality, while a 50% loss was considered the limit value of storability.

The results of sensory evaluation were analysed by statistical methods. The *t* test was used to analyse the changes caused by quick-freezing at —30 °C in fresh string beans and evaluated by comparative sensory tests. All the 9 bean varieties were subjected to this test.

The varieties subjected to storage test were examined at two months intervals by sensory evaluation. An analysis of variance was done with the results and the sensory values were interpreted by *Student*'s t test. The storage times required to reach 30% and 50% deterioration of flavour were obtained by calculation, since storage at —20 °C and —30 °C extended over 12 months only. The method of polynomials of the third order was used for calculation, proceeding from the presumption that the character of spoilage, as found in actual measurements, fits a curve of initially decreasing, then stagnating and finally again decreasing tendency.

The following equation (SVÁB, 1973) was used:

$$S = A + BM + CM^2 + DM^3 ,$$

where S = score ,

A, B, C, D = constants,

$$M = \frac{\text{months} - 6}{2} \quad \text{at} \quad -20 \text{ and } -30 \text{ °C} ,$$

$$M = \frac{\text{months} - 4}{2} \quad \text{at} \quad -10 \text{ °C} .$$

Data measured and calculated were graphically illustrated. Results were evaluated by analysis of variance and the least significant difference was determined.

$$LSD = t \sqrt{\frac{s^2 \cdot 2}{n}} ,$$

where LSD = the least significant difference (at 5%),
t = t value belonging to s^2,
s^2 = variance,
n = number of data used for calculating the average.

The rate of flavour deterioration was expressed as the reciprocal of the time needed to reach 30% and 50% flavour deterioration, respectively ($v = 1/t$), and plotted as a function of the logarithm of storage temperature (VAS, 1971).

2. Results

2.1. Dimensions

The length of the pods of the green and yellow string bean varieties studied was between 9 and 13 cm, their width between 9 and 11 mm. Results are the averages of 20 measurements, the standard deviation being 0—1.2 cm.

2.2. Results of colour tests

Results are shown in Tables 1 to 4. The tables contain the colour values characteristic of each variety, the characteristic wavelength, colour intensity and lightness value, as well as the standard deviation.

The colour of each bean variety was measured in the fresh state, after precooking for 2 min, after cooking for 15 min, quick-frozen at —30 °C and thawed. In addition the colour of the 3 varieties subjected to storage at 3 different temperatures was determined after 6 months. The latter samples were thawed and cooked for 10 min at 100 °C prior to colour testing.

As a general conclusion it may be established that the dominant wavelength dependent on the colour components of the individual variety presents, within limits, a basis of identification. However, within the individual variety the trend of change does not follow a definite pattern. While the wavelength λ_d characterizes the variety, at a given stage of ripeness, it is not sufficiently sensitive to indicate the effect of a treatment.

The saturation value (P_e) is not satisfactorily characteristic either of the colour changes in the bean pod upon treatment. It places the sample in the colour triangle and the darkening of the sample upon a treatment is shown by the increase of the saturation value but the saturation value is not sensitive enough to detect slight discolorations.

The lightness value (Y component), on the other hand, as a value independent of the wavelength, represents very satisfactorily darkening or fading of the sample. Bean pods of light yellow or green colour absorb a slight amount of light and reflect a great amount. Dark samples absorb more light and reflect less. The lightness factor expresses the amount of reflected light as percentage. Thus this value may be considered characteristic of bean varieties, even more of the changes occurring upon treatment or storage and therefore it may be taken into account as an objective quality characteristic.

These general observations are based on the results obtained with individual samples.

As seen in Table 1, the green bean varieties, *Carobel Wax* and *Jutta* became darker upon precooking, cooking and freezing. The light green original colour of the pods darkened upon these treatments. This is shown by the Y value. In varieties with green pods this change is of positive character, because the more saturated colour is more pleasing to the eye. The colour changes as occurring upon treatment in varieties with yellow pods may not be considered advantageous. The pods of originally vivid yellow colour turned brown upon cooking and freezing and this was shown by the reduced Y value. There was but a single variety with yellow pods which was not affected by either cooking or freezing and this was *Kinghorn Wax*. The colour of this variety was as pleasing after precooking, cooking and freezing as prior to these treatments.

Table 1

Colour of string bean varieties, in the fresh, cooked and quick-frozen state, as expressed in the C.I.E. system

Variety Treatment	$\overline{\lambda}_d$	$\overline{P}_e$	$\overline{Y}$	s_Y
Kinghorn Wax				
fresh, after harvesting	575	0.30	36.59	0.96
after precooking for 2 min	572	0.48	36.88	0.99
after cooking for 15 min	573	0.37	45.71	0.00
quick-frozen at −30 °C*	571	0.40	32.10	0.58
Wax Goldjuwel				
freshly picked	573	0.46	30.47	1.78
after precooking for 2 min	572	0.52	16.87	0.88
after cooking for 15 min	574	0.43	16.73	0.22
quick-frozen at −30 °C*	570	0.50	12.20	0.87
Carobel Wax				
freshly picked	565	0.32	27.34	0.73
after precooking for 2 min	568	0.27	11.05	2.45
after cooking for 15 min	570	0.34	17.79	0.82
quick-frozen at −30 °C*	567	0.35	18.65	0.60
Jutta				
freshly picked	567	0.39	30.14	3.92
after precooking for 2 min	564	0.34	19.07	0.75
after cooking for 15 min	572	0.38	10.11	1.05
quick-frozen at −30 °C*	565	0.40	13.20	0.95
Harvester (yellow)				
freshly picked	566	0.38	46.16	3.75
after precooking for 2 min	565	0.32	10.41	0.84
after cooking for 15 min	566	0.38	10.97	0.43
quick-frozen at −30 °C*	564	0.40	15.10	0.68
Juliska				
freshly picked	566	0.51	33.71	3.00
after precooking for 2 min	561	0.51	16.87	0.97
after cooking for 15 min	567	0.40	15.53	1.27
quick-frozen at −30 °C*	562	0.42	14.03	0.90

$\overline{\lambda}_d$ = average of dominant wavelength (nm)
P_e = average saturation
$\overline{Y}$ = average lightness (%)
s_Y = standard deviation (relative to Y)
* thawed (tested within one week of freezing)
Sample temperature was adjusted to 20 °C
All data represent three parallel measurements

Tables 2 to 4 give the results pertinent to the three green-pod varieties.

Table 2

Changes in colour of string bean variety Sanocrop during processing and frozen storage. C.I.E. characteristics

Treatment	$\bar{\lambda}_d$	$\bar{P}_e$	$\bar{Y}$	s_Y
Freshly picked	566	0.38	24.20	4.05
Precooked for 2 min	562	0.36	23.00	3.31
Cooked for 15 min	566	0.37	22.11	2.03
Quick-frozen at −30 °C, tested within 1 week of freezing after thawing	561	0.56	21.90	4.59
Stored for 6 months at −10 °C, thawed prior to testing	565	0.24	33.38	1.18
Stored for 6 months at −20 °C, thawed prior to testing	562	0.49	23.76	8.10
Stored for 6 months at −30 °C, thawed prior to testing	563	0.31	25.12	0.58
Stored for 6 months at −10 °C, thawed, cooked for 10 min	568	0.43	15.30	3.45
Stored for 6 months at −20 °C, thawed, cooked for 10 min	566	0.40	9.87	0.75
Stored for 6 months at −30 °C, thawed, cooked for 10 min	562	0.39	8.25	0.22

$\bar{\lambda}_d$ = average of dominant wavelength (nm)
$\bar{P}_e$ = average saturation
$\bar{Y}$ = average lightness (%)
s_Y = standard deviation (relative to Y)
All data represent three parallel measurements

With all the three varieties the colour became more saturated upon treatment. On comparing the colour of the stored samples to those immediately after quick-freezing, the lightness value seems to reflect satisfactorily the change in colour during storage at various temperatures. The lightness value for the 3 samples was higher after storage at —10 °C than immediately upon freezing. During storage for 6 months at this temperature the colour of the samples turned brownish. During storage at —20 and —30 °C the Y values were lower, fundamental changes were not observed and the results obtained with all three varieties were similar. The lightness value obtained after cooking showed similar trend. While the brownish colour of samples stored at —10°C was express, the colour of the samples stored at lower temperatures, with the exception of some spotty samples, turned a darker green.

As seen from the results the lightness value as measured with the *Lovibond* Tintometer seems adequate for the characterization of colour changes during storage (breaking down or transformation of chlorophyll, staining upon freezing).

Table 3

Changes in colour of string bean variety Harvester (green) during processing and frozen storage. C.I.E. characteristics

Treatment	$\bar{\lambda}_d$	$\bar{P}_e$	$\bar{Y}$	s_Y
Freshly picked	567	0.35	26.15	1.90
Precooked for 2 min	562	0.31	18.71	2.21
Cooked for 15 min	565	0.33	25.60	5.90
Quick-frozen at −30 °C, thawed for test within 1 week	561	0.28	12.12	0.58
Stored for 6 months at −10 °C, thawed prior to testing	566	0.39	19.28	12.70
Stored for 6 months at −20 °C, thawed prior to testing	566	0.45	16.13	4.13
Stored for 6 months at −30 °C, thawed prior to testing	565	0.48	15.46	2.01
Stored for 6 months at −10 °C, thawed, cooked for 10 min	566	0.39	12.02	1.00
Stored for 6 months at −20 °C, thawed, cooked for 10 min	568	0.39	9.34	0.33
Stored for 6 months at −30 °C, thawed, cooked for 10 min	568	0.40	6.17	0.25

$\bar{\lambda}_d$ = average of dominant wavelength (nm); $\bar{P}_e$ = average saturation; $\bar{Y}$ = average lightness (%); s_Y = standard deviation (relative to Y); All data represent three parallel measurements

Table 4

Changes in colour of string bean variety Budai zöld during processing and frozen storage. C.I.E. characteristics

Treatment	$\bar{\lambda}_d$	$\bar{P}_e$	$\bar{Y}$	s_Y
Freshly picked	566	0.37	9.29	0.87
Precooked for 2 min	559	0.28	4.09	0.80
Cooked for 15 min	557	0.17	7.71	0.37
Quick-frozen at −30 °C, thawed for test within 1 week	560	0.55	21.98	6.08
Stored for 6 months at −10 °C, thawed prior to testing	568	0.24	29.17	5.35
Stored for 6 months at −20 °C, thawed prior to testing	565	0.38	15.54	1.60
Stored for 6 months at −30 °C, thawed prior to testing	557	0.41	14.91	0.20
Stored for 6 months at −10 °C, thawed, cooked for 10 min	569	0.50	12.30	0.10
Stored for 6 months at −20 °C, thawed, cooked for 10 min	566	0.35	11.87	1.16
Stored for 6 months at −30 °C, thawed, cooked for 10 min	569	0.53	10.09	0.55

$\bar{\lambda}_d$ = average of dominant wavelength (nm); $\bar{P}_e$ = average saturation; $\bar{Y}$ = average lightness (%); s_Y = standard deviation (relative to Y); All data represent three parallel measurements.

2.3. Measurement of texture

The *Finometer* readings of the 9 string bean varieties and the changes occurring in their texture upon various treatments are given in Figs. 1 to 3. The texture of the fresh pods, after precooking for 2 min, after cooking for 15 min and after freezing at —30 °C was measured with each variety. Texture was also measured after storing the three varieties at —10 °C, —20 °C and —30 °C, resp., for 6 months.

The changes caused by the various treatments in the texture are well illustrated by the histograms. The most distinct change was brought about by cooking. The values obtained for all the 9 varieties upon cooking for 15 min were similar. The *Finometer* values of the samples precooked for 2 min, quick-

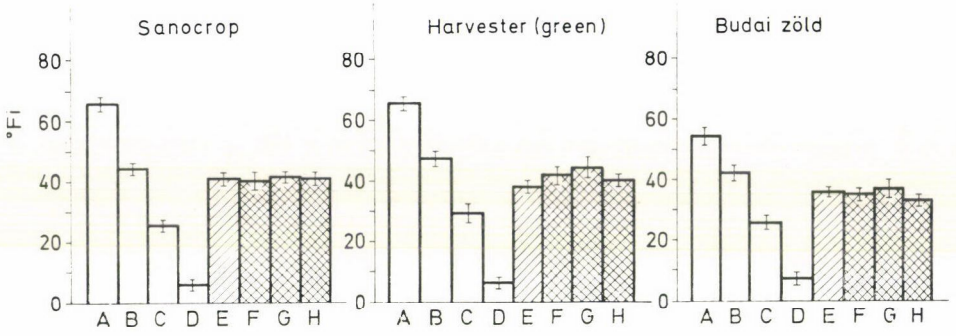

Fig. 1. Texture of some string bean varieties in *Finometer* degrees (°Fi). *A* — fresh; *B* — precooked for 2 min; *C* — precooked for 4 min; *D* — cooked for 15 min; *E* — precooked for 2 min and quick-frozen; *F* — precooked for 2 min, quick-frozen, stored at —10 °C for 6 months; *G* — precooked for 2 min, quick-frozen, stored at —20 °C for 6 months; *H* — precooked for 2 min, quick-frozen, stored at —30 C° for 6 months. Vertical bars indicate the standard deviation

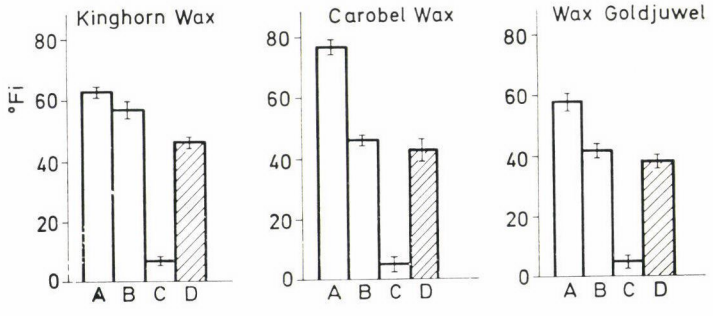

Fig. 2. Texture of some string bean varieties in *Finometer* degrees (°Fi). *A* — fresh; *B* — precooked for 2 min; *C* — cooked for 15 min; *D* — precooked for 2 min and frozen at —30 °C. Vertical bars indicate the standard deviation

frozen at —30 °C and thawed hardly differed from those obtained immediately upon precooking. Surprisingly, similar data were obtained after 6 months of storage.

The texture of the 9 varieties as affected by quick-freezing was tested also by regression analysis. When plotting the results (Fig. 4), the *Finometer* values pertinent to the fresh samples were shown on the abscissa and those of

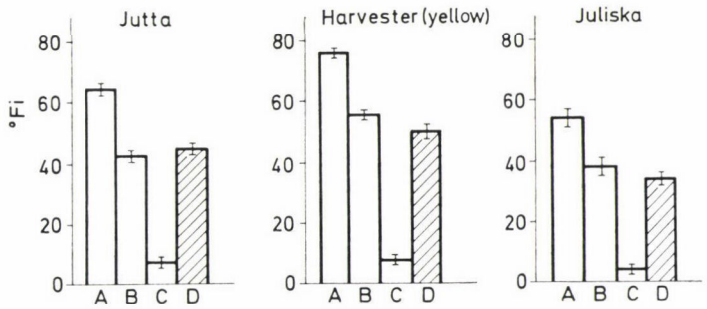

Fig. 3. Texture of some string bean varieties in *Finometer* degrees (°Fi). *A* — fresh; *B* — precooked for 2 min; *C* — cooked for 15 min; *D* — precooked for 2 min, frozen at —30 °C. Vertical bars indicate the standard deviation

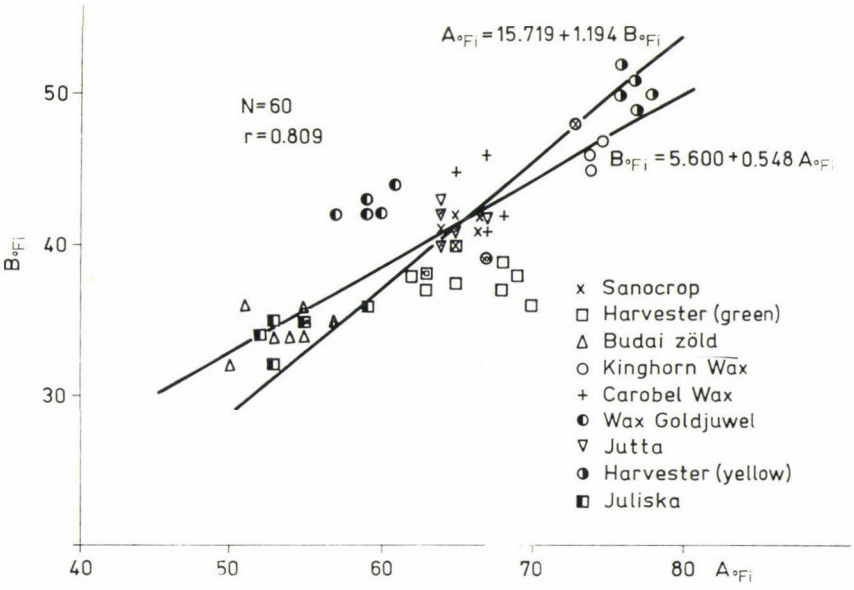

Fig. 4. Correlation between consistency values of fresh and frozen string beans. $A°Fi$ — *Finometer* values of the fresh beans; $B°Fi$ — *Finometer* values of the processed bean: precooked for 2 min; quick-frozen at —30 °C, thawed within a week of freezing to 20 °C

precooked and quick-frozen samples on the ordinate. The equations of the regression curves as calculated from the data:

$$A_{\circ Fi} = 15.719 + 1.194\, B_{\circ Fi}$$

and

$$B_{\circ Fi} = 5.600 + 0.548\, A_{\circ Fi}\,.$$

The value of the correlation coefficient:

$$r = 0.809.$$

The regression curve is nearly linear. The difference in the tissue structure of the various varieties is characterized by the correlation coefficient. The difference between the texture values upon quick-freezing is $\pm 16\,\%$.

The texture of the three varieties subjected to storage in the frozen state did not change substantially during storage as compared to that immediately upon quick-freezing.

2.4. Water-binding capacity

The effect of the various treatments and of storage was measured by the amount of juice released upon pressing under a definite load. The results as percentage of the sample weight are given in Table 5.

Table 5

Water-binding capacity of some string bean varieties as a function of processing and storage

Variety	Treatment					
	2' precooking		15' cooking		quick-freezing+	
	$\bar{X}$	s	$\bar{X}$	s	$\bar{X}$	s
Sanocrop	36.36	6.26	43.17	7.45	48.21	5.91
Harvester (green)	13.55	4.55	36.98	3.25	41.73	2.56
Budai zöld	25.61	7.61	38.38	12.34	37.84	7.90
Kinghorn Wax	21.55	5.76	29.25	0.26	34.50	6.30
Wax Goldjuwel	26.12	5.20	33.66	10.30	39.47	4.50
Carobel Wax	15.20	6.50	23.12	6.59	28.15	6.10
Jutta	19.35	4.30	26.37	3.90	35.25	5.20
Harvester (yellow)	11.13	3.44	26.51	7.20	32.15	6.15
Juliska	24.15	3.55	32.15	5.10	38.20	3.55

Table 5 (continued)

Variety	Storage (6 months) at					
	−10°C		−20°C		−30°C	
	$\bar{X}$	s	$\bar{X}$	s	$\bar{X}$	s
Sanocrop	49.62	5.10	49.10	4.8	48.5	6.5
Harvester (green)	42.50	4.30	41.90	3.7	42.0	1.5
Budai zöld	39.25	3.90	38.25	5.7	38.1	6.3

$\bar{X}$ = percentage juice release, average of 5 measurements
s = standard deviaton
+quick-freezing at −30 °C, tested within 1 week of freezing

The results permit of drawing conclusions as to the textural structure of the individual varieties. The varieties of higher juice percentage are more tender, juicier. Varieties of more solid structure release less juice. It is noteworthy that the values obtained for the varieties were nearly the same after 6 months of storage as immediately upon quick-freezing. It may be concluded from the table that juice release, measured under identical conditions, depends largely on variety.

2.5. Vitamin C content

The vitamin C content was determined as described under para. 1.4.4. in detail.

After a storage period of 10 months the samples stored at —10 °C lost completely their vitamin C content. The samples stored at —20 °C had a measurable amount of vitamin C and those stored at —30 °C about twice as much.

The results obtained by visual evaluation of chromatograms of individual varieties stored at different temperatures are shown in Table 6.

Table 6

Vitamin C content of some bean varieties as a function of storage temperature after 10 months of storage (in ppm)

Variety	Storage temperature					
	—10 °C		—20 °C		—30 °C	
	$\bar{X}$	s	$\bar{X}$	s	$\bar{X}$	s
Sanocrop	0	0	4.0	0.80	10.0	0.95
Budai zöld	0	0	4.0	0.50	8.5	0.70
Harvester (green)	0	0	5.0	0.70	8.0	0.80

$\bar{X}$ = average vitamin C content, 3 measurements
s = standard deviation
Results relate to 100 graw material

2.6. Sensory evaluation

For sensory evaluation the fresh samples were cooked for 15 min, while those quick-frozen or stored for 10 min. Each sample was scored in two parallel tests by the same panel of 5 members.

The sensory evaluation served to compare the varieties in the fresh state and in the quick-frozen state and follow up changes occurring upon quick-freezing. With 3 varieties (samples of 1972) the effect of storage, the keeping quality as a function of flavour score and the rate of deterioration were also studied. The spoilage rate of the varieties *Sanocrop*, *Harvester (green)* and *Budai zöld* was studied at 3 different storage temperatures.

2.6.1. Effect of quick-freezing upon sensory value. Sensory values obtained for the fresh beans and after quick-freezing at —30 °C are compared in Table 7. The quick-frozen samples were tested within one week subsequent to freezing.

As seen from the Table 7, the colour of green bean varieties was significantly better after quick-freezing. This is probably due not only to the interruption of enzyme activity. The fresh bean samples were also cooked for 15 min prior to evaluation. Thus, it may be stated that quick-freezing has an advantageous effect on the green colour of the pods. The colour of the bean varieties with yellow pods distinctly deteriorated upon quick-freezing and was significantly poorer after freezing.

The effect of quick-freezing upon the odour of both the green and yellow varieties was uniform, inasmuch as the characteristic odour diminished and, instead, the well-known hay scent appeared. Though this odour change was not very intense, the result was significant, except for the *Budai zöld* variety which was scored low even before freezing. These observations were made within one week of freezing.

As regards the flavour, significant difference was found only with the yellow variety *Wax Goldjuwel*. The flavour scores were lower for almost every variety, however, the difference was not significant.

The change in texture upon freezing did not show a definite trend. Significant difference was found only with the variety *Juliska*.

2.6.2. Sensory changes during storage. The bean varieties purchased in 1972 were stored at —10, —20 and —30 °C, respectively. They were subjected to sensory evaluation at two-month intervals. The colour, odour, flavour and texture were scored, and the scores for flavour are analysed below.

The flavour scores were evaluated by the t test. The results of analysis, the average flavour scores and the standard deviations are given in Table 8.

The flavour score of the samples was studied as a function of the shelf-life, taking into account 30% and 50% deterioration. On reaching 30% deterioration the sample drops below grade 1. Deterioration beyond 50% signifies unpalatable quality. The flavour values measured and calculated as described in para. 1.5. are illustrated in Figs. 5—7.

Table 7

Sensory evaluation of some bean varieties freshly picked and after quick-freezing

Variety	Treatment	Colour			Odour			Flavour			Texture		
		$\bar{X}$	s	t	$\bar{X}$	s	t	$\bar{X}$	s	t	$\bar{X}$	s	t
Sanocrop (green) (1972)	*A*	3.9	0.50	2.43	4.1	0.65	2.78*	4.5	0.50	2.32	4.0	0.79	1.80
	B	4.1	0.65		3.6	0.42		3.8	0.27		4.0	1.00	
Harvester (green) (1972)	*A*	3.7	0.45	6.06**	4.3	0.57	2.78*	4.4	0.65	2.43	4.0	0.75	2.05
	B	4.3	0.27		4.0	0.30		4.2	0.27		4.4	0.65	
Budai zöld (green) (1972)	*A*	2.7	0.67	3.47*	3.6	0.22	2.05	3.8	0.45	3.45*	2.8	0.45	1.89
	B	4.3	0.76		3.6	0.74		3.4	0.42		3.8	0.55	
Kinghorn Wax (yellow) (1973)	*A*	4.6	0.42	2.78*	4.6	0.42	3.27*	4.6	0.42	1.27	4.8	0.45	1.00
	B	3.9	0.55		4.3	0.45		4.2	0.27		5.0	0.00	
Wax Goldjuwel (yellow) (1973)	*A*	3.7	0.45	3.27*	4.5	0.50	3.25*	4.5	0.87	2.89*	4.8	0.45	1.51
	B	3.4	0.42		3.9	0.22		3.9	0.74		4.7	0.45	
Carobel Wax (green) (1973)	*A*	3.8	0.84	6.55**	4.8	0.45	3.45*	4.0	0.00	0.55	5.0	0.00	1.69
	B	4.6	0.42		4.1	0.22		4.2	0.27		4.8	0.27	
Jutta (green) (1973)	*A*	3.4	0.82	2.07	4.7	0.45	3.25*	4.3	0.27	2.23	4.8	0.45	1.00
	B	4.1	0.22		4.5	0.35		4.5	0.35		5.0	0.00	
Harvester (yellow) (1973)	*A*	4.9	0.10	9.12***	5.0	0.00	6.84**	5.0	0.00	1.69	4.9	0.20	2.16
	B	4.0	0.30		4.8	0.20		4.8	0.20		4.7	0.30	
Juliska (yellow) (1973)	*A*	4.9	0.10	6.84**	4.8	0.10	3.25*	5.0	0.00	2.23	5.0	0.00	2.56
	B	4.1	0.15		4.2	0.20		4.6	0.80		4.8	0.10	

A = freshly picked samples cooked for 15 min

B = quick-frozen at −30 °C, tested within 1 week after thawing and cooking for 15 min

$\bar{X}$ = average scores (5 tests)

s = standard deviation

t = value according to *Student*

* difference significant at the $P_{95\%}$ level

** difference highly significant at the $P_{99\%}$ level

*** difference very highly significant at the $P_{99.9\%}$ level

Table 8

Effect of storage time and temperature on the flavour of some string bean varieties during frozen storage

Variety	Storage time (months)	Storage prior to freezing		Storage temperature (°C)								
				−10			−20			−30		
		$\bar{X}$	s	$\bar{X}$	s	t	$\bar{X}$	s	t	$\bar{X}$	s	t
Sanocrop	0	4.5	0.50	—	—		—	—		—	—	
	2			3.40	0.22	5.00**	4.10	0.22	1.81	4.20	0.27	1.25
	4			3.30	0.27	5.00**	3.70	0.45	2.85*	3.90	0.22	2.72
	6			2.40	0.42	7.50**	4.00	0.35	1.92	4.20	0.27	1.25
	8			—	—		3.90	0.42	2.14	4.10	0.22	1.81
	10			—	—		3.90	0.42	2.14	4.10	0.22	1.81
	12			—	—		3.80	0.27	2.91*	4.20	0.45	1.07
Harvester (green)	0	4.4	0.65	—	—		—	—		—	—	
	2			3.60	0.55	2.16	4.20	0.45	0.58	4.20	0.45	0.58
	4			2.90	0.22	5.00**	3.90	0.42	1.51	3.90	0.22	1.66
	6			2.40	0.22	6.66**	3.80	0.45	1.76	4.30	0.27	0.33
	8			—	—		3.90	0.65	1.25	4.20	0.45	0.58
	10			—	—		3.60	0.55	2.16	4.10	0.55	0.81
	12			—	—		3.30	0.45	3.23*	3.90	0.42	1.51
Budai zöld	0	3.8	0.45	—	—		—	—		—	—	
	2			2.70	0.45	3.92*	3.70	0.45	0.35	3.70	0.42	0.35
	4			3.10	0.22	3.50*	3.70	0.57	0.31	3.70	0.45	0.35
	6			2.00	0.79	4.50*	3.00	0.35	3.33*	3.40	0.22	2.00
	8			—	—		3.00	0.45	2.85*	3.20	0.35	2.50
	10			—	—		2.70	0.42	4.23*	3.00	0.61	2.42
	12			—	—		2.70	0.50	3.92*	3.00	0.61	2.42

The samples were cooked for 15 min prior to testing
$\bar{X}$ = average score (5 tests)
s = standard deviation
t = t value according to *Student*

* difference between the fresh and quick-frozen sample significant at the $P_{95\%}$ level
** difference between the fresh and frozen sample highly significant at the $P_{99\%}$ level

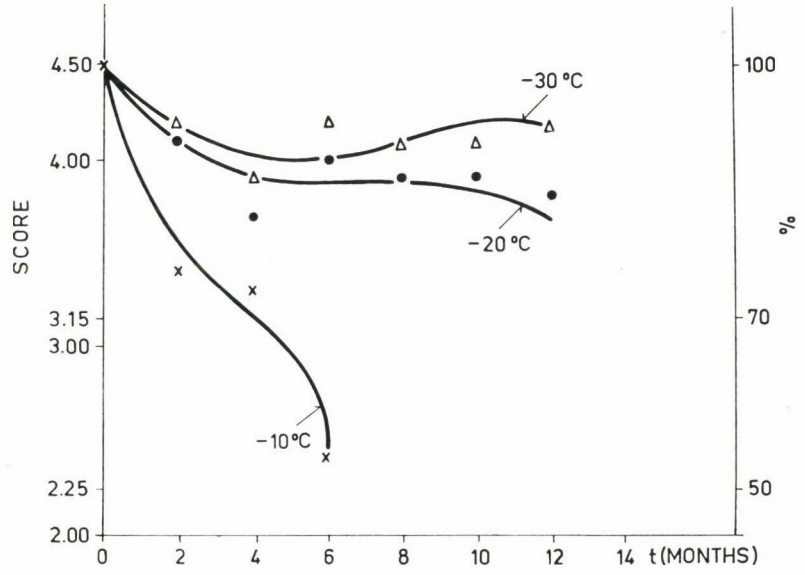

Fig. 5. Flavour deterioration of the string bean variety *Sanocrop* as a function of storage time (t). Experimental points and calculated curves

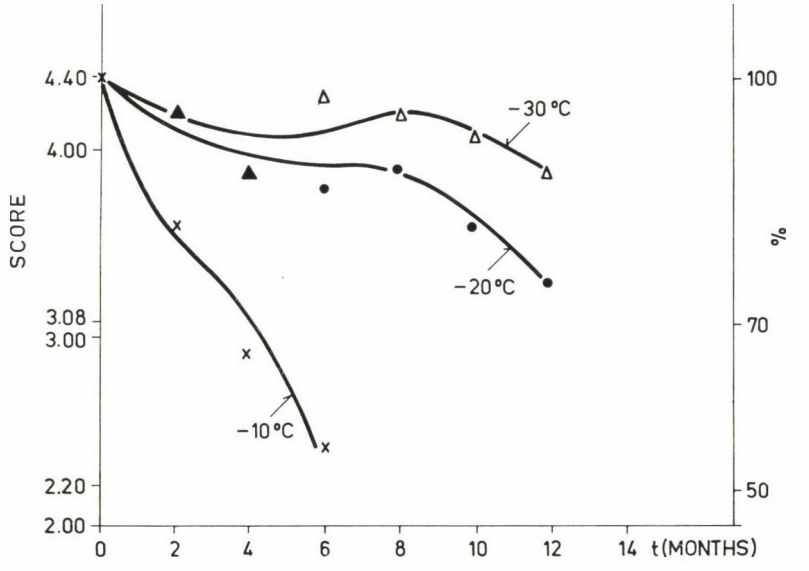

Fig. 6. Flavour deterioration of the string bean variety *Harvester (green)* as a function of storage time (t). Experimental points and calculated curves

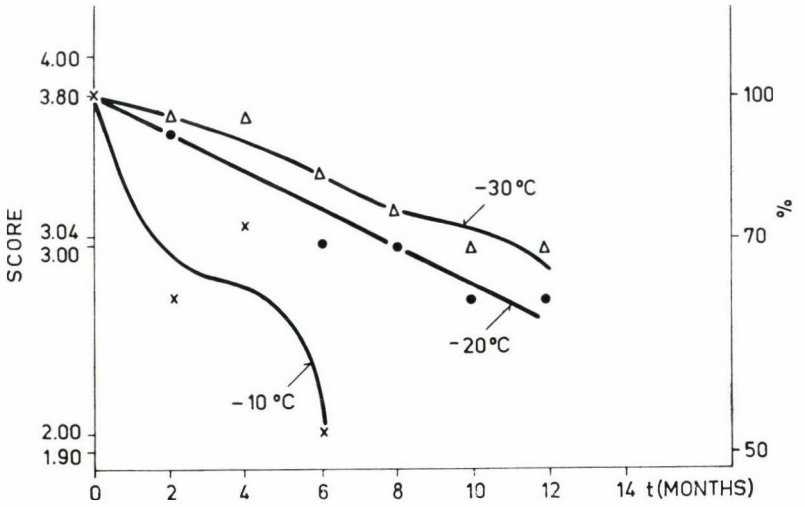

Fig. 7. Flavour deterioration of the string bean variety *Budai zöld* as a function of storage time (t). Experimental points and calculated curves

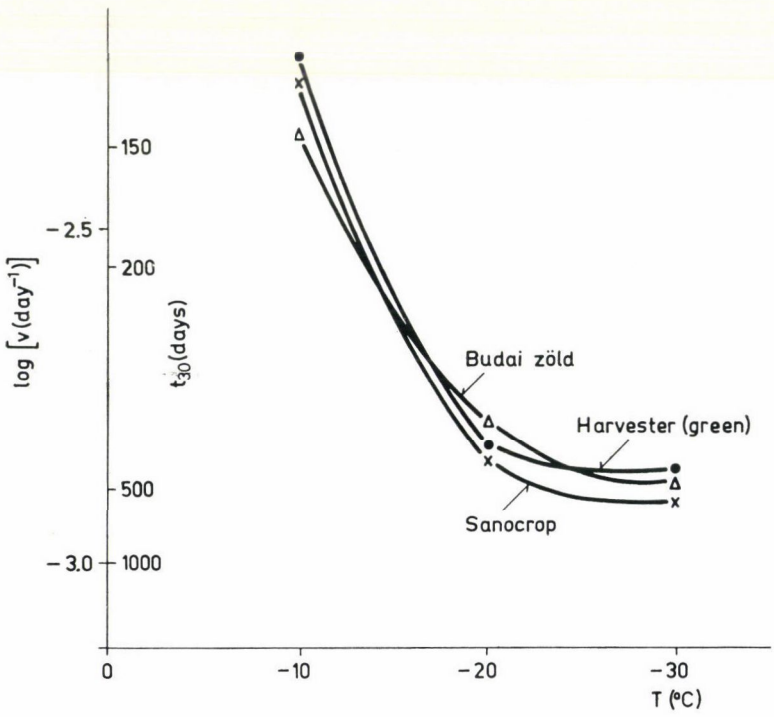

Fig. 8. Rate of deterioration (v, day^{-1}) and keeping time related to 30% deterioration of flavour (t_{30}) as a function of storage temperature (T)

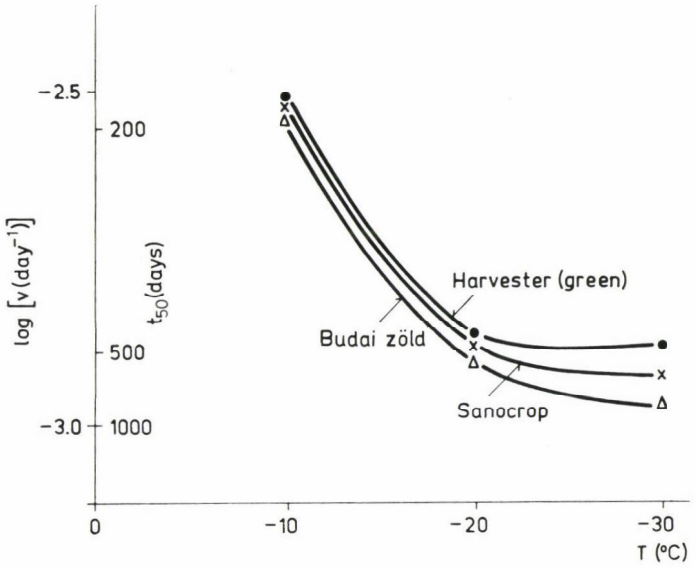

Fig. 9. Rate of deterioration (v, day^{-1}) and keeping time related to 50% deterioration of flavour (t_{50}) as a function of storage temperature (T)

Figure 5 shows the flavour deterioration of the *Sanocrop* bean variety.

As seen, the course of change in flavour score depended on the storage temperature. At —10 °C the predetermined limits of deterioration were reached rapidly. At —20 °C, during the time controlled, the period of rapid deterioration was reached, while at —30 °C only the period of stagnation prevailed.

A similar trend was observed with the *Harvester* variety as shown in Fig. 6.

Since with the above two varieties deterioration at lower temperatures proved slow, the polynomial of third order, adapted in view of the presumed deterioration period, permitted an approximation only.

The flavour deterioration curve of the *Budai zöld* variety did not follow the same course.

As seen, the shape of the curve is nearly linear. Therefore in calculating the shelf-life, only linear members of the equation were taken into account.

The rate of deterioration is illustrated in Figs. 8 and 9.

The curve of all varieties took a similar course.

The results of the statistical evaluation of keeping times of the three varieties are shown in Table 9.

As can be seen in the Table 9 and in the graphs, the initially better varieties [*Harvester (green)*, *Sanocrop*] reached the threshold of deterioration more rapidly than the variety with the lowest initial flavour score. Thus, *Budai zöld*

Table 9

Keeping time of frozen string beans stored at various temperatures

Variety	Storage temperature					
	−10 °C		−20 °C		−30 °C	
	average number of days needed to reach deterioration levels of					
	30%	50%	30%	50%	30%	50%
Sanocrop	132	188	420	483	534	594
Harvester (green)	126	185	387	456	432	478
Budai zöld	147	193	339	552	468	764

which received the lowest value in the initial test, reached the threshold for a predetermined percentage of deterioration more slowly. This, of course, was a consequence of the fact that, in the latter case, a low initial flavour score served as the basis for comparison against which deterioration was measured.

Flavour scores of the three varieties stored were also evaluated by analysis of variance. The LSDs for keeping times at 30% and 50%, resp., were 79 and 163 days, resp. Thus, no significant difference could be detected between keeping times of the varieties stored at identical temperatures. Significant differences could, however, be demonstrated between keeping times of the same variety, as measured at various temperatures.

On the basis of flavour scores obtained for the fresh and quick-frozen beans, *Carobel Wax* and *Jutta* seemed suitable for freezing. The colour of varieties with yellow pods turned brown upon quick-freezing, therefore these varieties are recommended for canning. They proved to be of very good quality on the basis of their flavour and texture scores after cooking and even after quick-freezing.

3. Conclusions

The results of the tests show that the colour of green string beans improved, while that of the yellow varieties deteriorated upon quick-freezing. As regards flavour, results are not uniform. Taking into account the colour and flavour changes the following varieties are suitable for quick-freezing:

Sanocrop,
Harvester (green),
Carobel Wax,
Jutta.

In the storage tests all three varieties: *Sanocrop, Harvester (green)* and *Budai zöld,* proved to be suitable for storage. However, the use for freezing of

the *Budai zöld* variety is not recommended, because of its low initial flavour value.

With storage at —20 °C, the shelf-life (till 30% flavour deterioration) of the samples was 10—16 months, at —30 °C it was found to be about 18 months.

The texture of the samples did not seem to deteriorate during storage. The *Finometer* values did not decrease during storage. The same conclusion was drawn from the water-binding capacity tests.

From the point of view of the vitamin C content storage at —30 °C proved to be the most advantageous.

*

The author wishes to thank Dr. M. DEMECZKY, Head of Department, for his valuable advice and Miss I. KALÁSZ for her devoted technical assistance.

The author is indebted to E. ZUKÁL, Head of Department, INSTITUTE FOR CHEMISTRY AND FOOD ANALYSIS (KÉVI), and Miss I. LENDVAI for their assistance in the mathematical processing of the sensory scores.

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Acta Alimentaria, Vol. 4 (3), pp. 251–269 (1975)

INVESTIGATION INTO THE PERMEABILITY OF POLYMER MEMBRANES OF FOOD PACKAGING QUALITY TO GASES AND WATER VAPOUR AFTER RADIATION TREATMENT WITH RADURIZING DOSES

I. VARSÁNYI

(Received March 25, 1974)

This work involved the determination of permeability to nitrogen, oxygen, carbon dioxide and water vapour during storage, combined preservation methods and up-to-date packaging in a controlled atmosphere by means of the method developed by the author (Figs. 1, 2 and 3) at the temperature of 30 °C, from 0 to 35 days after treatment with doses of 50, 100, 200, 400 and 800 krad, resp. Since permeability is closely related to the ratio of the ordered crystalline regions to the amorphous region in the membrane which latter, from the point of view of permeability, can be considered an active region, the results obtained by the determination of permeability were checked by determining the crystalline to amorphous ratio in the polymers by photometry in the infra-red region. In addition, these results also provided information for the assessment of the membranes from the hygienic point of view. The results were statistically evaluated and compared.

It was found that the permeability of low-density polyethylene (Fig. 4), high density polyethylene (Fig. 5), polypropylene (Fig. 6), polyvinylchloride (Fig. 7), polyvinylidene chloride (Fig. 8) and polyester (Fig. 9) to nitrogen, oxygen, carbon dioxide and water vapour after radurization did not differ significantly from the permeability of these polymers prior to radiation treatment. Heat treatment, on the other hand, caused highly significant differences between the permeabilities of treated and untreated polymers.

These findings were confirmed by the results of the determination of the ratio of the crystalline to the amorphous region (Figs. 10 to 15). There was no significant difference between the results of IR photometry performed on radiation treated polymers and the results obtained with untreated polymers. This indicated not only the lack of a detectable difference in the ratio of the amorphous region as a result of irradiation, but also a favourable behaviour of the polymers from the hygienic point of view. Consequently radurization with 800 krad and lower doses can be performed also on packaging and protecting films in direct contact with food.

Summarizing the results: irradiation with radurizing doses caused no change in the mechanism of gas and vapour permeation through the polymers under investigation, thus all correlations and statements concerning permeability which are generally valid for polymer membranes can be applied also to polymers treated with gamma-rays.

In the intensive research of the peaceful application of ionizing radiations their applicability in the food industry occupies an important place. Results obtained in Hungary and abroad have proven that ionizing radiations can advantageously be applied to improve storage, preservation and quality in general, primarily where traditional methods of treatment can no longer be used with the same results.

Researchers engaged in the study of the changes caused by radiation treatment in plastics used by the food industry applied gamma-radiation with

the primary aim to improve some property — usually the mechanical strength — of the polymer.

Doses capable of modifying the properties of polymers are, as a rule, several orders higher than the dose level used for the pasteurization of foodstuffs. Thus, researchers interested in the modification of polymers studied both theoretically and practically only considerably higher doses of radiation.

Research into the application possibilities of ionizing radiation to the food industry necessitated, however, the investigation of the effect of radurization, that is of "pasteurizing" doses on the permeability of polymer membranes used by the food industry.

The aim of our investigations was to establish the influence of radurization doses of ionizing gamma-radiations on the permeability to gases and water vapour of plastic protective and coating films of the most favourable properties from the point of view of storage and preservation.

1. Materials and methods

1.1. Materials

The tests were carried out on polymer membranes which are the most widely used as protective and coating materials in the food industry. The model substances to which the radiation treated polymer membranes were exposed were chosen on the grounds of practical technological considerations.

1.1.1. Polymer membranes. Of the low-density polyethylene films the one manufactured by the HUNGÁRIA MŰANYAGFELDOLGOZÓ VÁLLALAT, Budapest, Hungary, was tested. The 0.0040 cm thick film has a density of 0.91 g cm^{-3} and it is widely used for the most diverse purposes.

High-density polyethylene was the product of THE METAL BOX Co., had a density of 0.95 g cm^{-3} and a thickness of 0.0038 cm. This type of product seems to be appropriate for up-to-date heat and radiation preservation procedures.

The polypropylene membrane was a 0.0040 cm thick film of 0.90 g cm^{-3} density, manufactured by MONTECATINI EDISON. Since the material is translucent and can be sterilized, it is suitable for special purposes.

The polyvinylchloride in compliance with the food-hygienic specifications came from US RUBBER Co. in the form of a 0.0030 cm thick film of 1.38 g cm^{-3} density.

Polyvinylidene chloride manufactured by AB ÅKERLUND & RAUSING was a 0.0030 cm thick film of 1.57 g cm^{-3} density. Its favourable shrinking properties make the material particularly suitable for packaging fresh vegetables and fruits.

Of the polyester membranes the 0.0020 cm thick poly(terephthalic acid glycol ester) type film of 1.40 g cm^{-3} density, manufactured by KALLE AG was tested. Due to its translucency and good shrinking properties this film is much favoured in the food industry.

1.1.2. Permeating model substances. The nitrogen gas complied with the specifications of HUNGARIAN STANDARD MSZ No. 1605—69 and was of "high purity", *i.e.* of a purity of 99.995% (v/v).

The purity of the oxygen gas was 99.5% (v/v) in compliance with the specifications of HUNGARIAN STANDARD MSZ No. 1604—69.

The quality of the carbon dioxide gas complied with the specifications of HUNGARIAN STANDARD MSZ No. 20915—68, its purity was 99.5% (v/v).

The water vapour used as model substance came from the saturated vapour space above the surface of twice distilled water.

1.2. Methods

Radiation treatment proceeded according to the usual method of the CENTRAL FOOD RESEARCH INSTITUTE, Budapest, Hungary.

For the determination of permeability a method was worked out on the grounds of our earlier experience and literature data and was finally found suitable to measure very small quantities of permeated materials. Since there is a close correlation between permeability and the structure of the polymer as pointed out by RAINE and co-workers (1945), MORGAN (1953), WALTER and REDING (1956), MYERS and co-workers (1957), BENT (1957), ALTER (1962) and KWEI and ARNHEIM (1964), the effect of radiation treatment was studied with the help of IR spectroscopy.

The results were statistically evaluated.

1.2.1. Radiation treatment. The samples were irradiated with the ^{60}Co gamma-radiation source of 45 kCi activity in the CENTRAL FOOD RESEARCH INSTITUTE at temperatures between 10 and 15 °C and a 200 krad per hour dose rate. The absorbed doses were 50, 100, 200, 400 and 800 krad, resp.

The doses absorbed by the samples were measured according to WEISS and co-workers (1955) by means of chemical dosimetry.

1.2.2. Determination of the permeability to gases. The principle of the method is as follows: the gas is led into the adsorption space of a special thermostated diffusion cell divided into two compartments by the membrane under investigation, so that the gas passing through the membrane reaches the desorption space from where it is carried by the carrier gas into a sensing device suitable for the detection of the quantity of gas which had passed through the membrane. The sensing device is the gas chromatograph. By means of this arrangement the entire process of permeation can be followed, so that not only the amounts of gases pertaining to the state of equilibrium can

be measured, but also the quantities which passed through the membrane at the beginning of the process. The diagram in Fig. 1 shows the measuring system.

The apparatus consists of the following main parts: thermostated diffusion cell (*1*), gas chromatograph connected to a compensator and a recorder (*2*), gauge, indicating the pressure difference between the gas in the adsorption

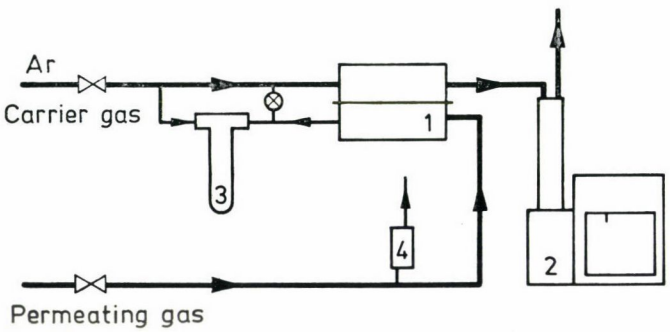

Fig. 1. Diagram of the apparatus for the determination of the permeability to gases. *1* — thermostated diffusion cell; *2* — gas chromatograph connected to the compensator; *3* — device signalling the pressure difference between the adsorption and desorption spaces of the diffusion cell; *4* — pressure difference stabilizer

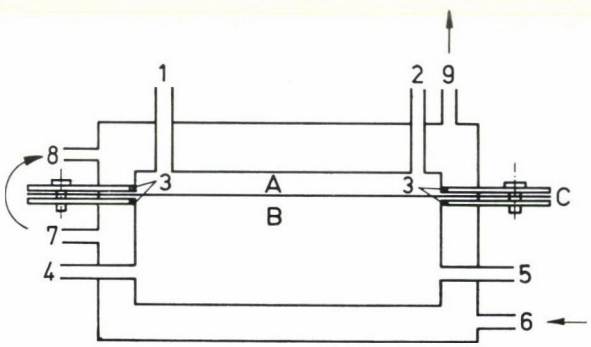

Fig. 2. Diagram of the diffusion cell used for the determination of permeability. *A* — adsorption space; *B* — desorption space; *C* — membrane. *1* — inlet for the gas or vapour to be measured; *2* — tube joined to the pressure difference signalizer and equalizer; *3* — gasket rings; *4* — carrier gas inlet; *5* — carrier gas outlet; *6* and *8* — inlets for the heating or cooling fluids by which the desired temperature is ensured; *7* and *9* — outlets for the same fluids

space and the gas in the desorption space of the diffusion cell (*3*), pressure equalizer (*4*).

The diffusion cell was constructed by utilizing the already described theoretical concepts and practical experience. The sketch of the measuring cell is shown in Fig. 2.

As shown in Fig. 2 the measuring cell has a jacket and is divided into

two parts by the membrane to be investigated. The diffusing gas or vapour enters space *A* through tube *1* at the desired moment and diffusing through membrane *C* reaches desorption space *B*. From here the desorbed molecules are carried through pipe *5* by the carrier gas entering through pipe *4* into the gas chromatograph. The short tube *2* is intended to equalize the pressure, while the hard rubber rings *3* serve for the hermetic closure of the cell.

The jacket of the apparatus enables the accurate adjustment of the temperature, so that the heating or cooling liquid reaches the jacket from the thermostat through tube *6* and leaves it through tube *7* at the bottom of the jacket. It is recirculated by tube *8* into the upper part of the jacket and is led through tube *9* back into the thermostat. If the difference between the temperature of the experiment and the ambient temperature is great, further if the pipe-line between the thermostat and the diffusion cell is so long as to cause a heat loss greater than the fluctuations of the thermostat, in the interest of accurate temperature adjustment it might be expedient to control the relay of the thermostat by a heat senser directly built into the diffusion cell. The upper and lower parts of the cell are clamped together with 6 bolts to make it gas-tight.

The tubular column gas chromatograph used in the experiments was manufactured by W. G. PYE AND CO., LTD. (Cambridge). The experiments were carried out at 150 °C detector temperature and 1 500 V detector voltage, using argon as carrier gas at a flow rate of 50 cm^3 per minute.

The inaccuracy of the gas chromatograph with respect to the amplitude was 5%, with respect to the end-value of the HONEYWELL type compensograph connected to it, it was 0.5%. With this arrangement 10^{-3} to 10^{-6} cm^3 of gas per cm^3 of carrier gas could be determined per second.

Since diffusion rate is an exponential function of the temperature, it was essential to keep the temperature at a constant value during measurements. Temperature was controlled by an ultrathermostat using water as heat transfer medium. The reliability of the switch which regulated the heating of the ultrathermostat was ± 0.1 °C.

A suitably constructed, mercury-filled capillary was used to signal the gas pressure differences between the adsorption and desorption spaces of the diffusion cell, while gas pressure was equalized, *i.e.* stabilized by a specially constructed, also mercury-filled pressure-equalizing device with adjustable counter-pressure. The two devices together served at the same time as protection against any possible over-pressure in the apparatus.

The measurements were carried out at 30 °C in 5 replicates.

1.2.3. Determination of water vapour permeability. The diagram of the apparatus constructed for the determination of the water vapour permeability of polymer films after treatment with radurizing radiation doses is shown in Fig. 3.

The apparatus consists of the following parts: device containing phosphorus pentoxide for the drying of the carrier gas (*1*), pressure equalizer (*2*), diffusion cell (*3*), instrument for measuring the amount of vapour which has passed through the membrane (*4*), gauge measuring the quantity of carrier gas (*5*).

The diffusion cell is the same as the one described above. The quantity of water vapour which diffused through the membrane was determined by means of the *Moisture Monitor* type 26—393 of the firm BELL AND HOWELL, GMBH, used generally for the detection of water vapour in gases. This appa-

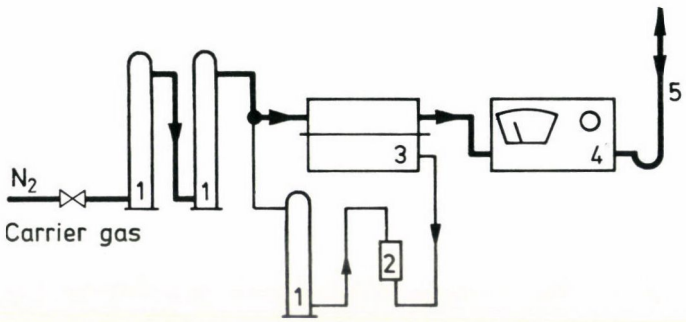

Fig. 3. Diagram of the apparatus used for the determination of permeability to water vapour. *1* — drying column packed with phosphorus pentoxide to remove all traces of water from the carrier gas; *2* — pressure equalizer; *3* — diffusion cell; *4* — device to measure the quantity of diffused water vapour; *5* — flowmeter

ratus signals the quantity of water vapour on the principle of electrical conductivity, that is of electric dialysis. The phosphorus pentoxide adsorbed between platinum wires of known resistance and kept under known voltage binds the water in the gas passing through the apparatus, so that a current will flow between the very closely situated platinum wires, while the phosphorus pentoxide is regenerated and the water decomposed into hydrogen and oxygen. These latter are then removed by the carrier gas. Since the resistance of the measuring cell changes as a function of the quantity of adsorbed water on the phosphorus pentoxide, the quantity of water vapour in the unit volume of gas passing through the system can directly be read off a suitably calibrated ammeter.

The measurements were carried out by placing the cleaned and dried membrane into the diffusion cell (*3*) (Fig. 3) whose temperature was adjusted and controlled with the help of an ultrathermostat using water as heat transfer medium.

The flow of the carrier gas was then started through a pressure reducing valve mounted on the gas cylinder. Any traces of water which might possibly interfere with the measurements were removed from the carrier gas prior to

entering through the diffusion cell the instrument, by allowing it to flow through 50 cm high, phosphorus pentoxide filled columns of 5 cm internal diameter. Drying of the system was continued till the sensing instrument signalled a zero water vapour concentration which meant at the same time that the entire system was free of disturbing traces of water vapour.

Next, twice distilled water was introduced into the adsorption side of the diffusion cell through inlet (*1*) (Fig. 2) and the carrier gas flowing into the desorption space (*4*) and leaving from there through outlet (*5*) — now containing the water molecules which had passed through the membrane — was introduced into the sensing instrument (*4*) (Fig. 3) whose needle showed a deflection proportional to the concentration of the water vapour in the carrier gas. Since the sensing device was factory-calibrated into water vapour concentration units (in ppm), the permeability constant could be determined from the quantity of water vapour passing through the membrane in unit time — as indicated by the needle of the instrument —, the thickness of the polymer membrane and the size of its active surface being known.

Pressure differences between the adsorption and desorption spaces were equalized by a specially constructed mercury pressure equalizer. The gas mixture leaving the instrument for the determination of the quantity of water vapour was let out into the open through a device measuring the quantity of gas flow.

The flow rate of the nitrogen gas used as carrier was 100 $cm^3\ min^{-1}$, the reliability of the instrument which measured the quantity of water vapour was, with reference to the total deflection of the needle, $\pm 5\%$. The reliability of the switch of the ultrathermostat which regulated the temperature of the diffusion cell was ± 0.1 °C. Since the quantity of water vapour which flowed through the membrane could directly be read in ppm concentration units from the scale of the instrument, the calibration of the latter was not necessary. Depending on the permeability of the membrane the duration of a single measurement varied between 20 and 138 minutes.

The water vapour permeability constant of polymer membranes treated with 0, 50, 100, 200, 400 and 800 krad doses, respectively, was determined at 30 °C. Five measurements were performed at this temperature.

1.2.4. Determination of the crystalline part. Investigation of the changes induced by radiation treatment in the crystalline to amorphous ratio of polymers had a twofold objective. On the one hand, the aim was to check the results obtained by permeability measurements by another method, while it appeared desirable from the point of view of food hygiene to find out whether radiation treatment has caused a transformation in the structure of the polymer leading perhaps to degradation.

The ratio of the crystalline part in polyethylenes was calculated on the basis of the work of NICHOLS (1954), HENDUS and SCHNELL (1961), KOCHKINA

(1968), and DIETL (1969) from the intensities of the absorption bands at 1 299 cm^{-1} and 1 887 cm^{-1} wavenumbers.

Polypropylene was studied according to the evaluation method of ABE and YANAGISAWA (1959), BRADER (1960), LUONGO (1960), MIYAZAWA (1964) from the intensity quotient for the bands at 973 cm^{-1} and 998 cm^{-1}.

In the case of polyvinylchloride the syndyotactic ratio, *i.e.* the crystalline part of the polymer was determined in agreement with the work of BURLEIGH (1960), ENOMOTO (1961), SHIPMAN and co-workers (1962), PRESTON and LAWSON (1964) and KRIMM (1965) from the value of the optical density quotient at 603 cm^{-1}/634 cm^{-1} as characteristic of the syndyotactic/isotactic ratio.

With respect to polyvinylidene chloride changes in the vinyl chloride/vinylidene chloride ratio were determined on the basis of the work of ELLIOT (1959), NARITA and co-workers (1959), KRIMM (1960) and GROEBEL (1968) with the help of the optical density quotient pertaining to the 1 206 cm^{-1}/1 255 cm^{-1} wave-bands.

The ratio of the ordered crystalline part to the amorphous part in polyester was investigated according to the suggestions of COBBS and BURTON (1953), GRIME and WARD (1958), MIYAKE (1959), ZACHMANN and STUART (1961) and HEFFELFINGER and SCHMIDT (1965). In the crystalline configuration the two hydrogen atoms on two neighbouring carbon atoms of ethylene glycol, which links the aromatic rings with an ester-type bond, are in a *trans*-position. For the determination of the ratio of the *trans*-isomer the value of absorption at 975 cm^{-1} was related to the absorption value at 794 cm^{-1}. The effect of radiation treatment was determined from the correlation between specific density and crystal content published in the literature.

The measurements were performed with carefully cleaned strips of films at room temperature and at atmospheric moisture content, using the UNICAM SP 200 and CARL ZEISS, JENA, UR 10 recording spectrophotometers.

1.2.5. Evaluation of the results. The results were statistically evaluated. The distribution of the standard deviation of the average values calculated from the experimental results was checked by means of the *Bartlett* test and if found to originate from a homogeneous population, the average values were compared by analysis of variance (SVÁB, 1967).

The calculations were performed on the OLIVETTI PROGRAMMA 101 and HEWLETT-PACKARD 9100 B type table computers.

2. Results

The effect of treatment with food pasteurizing (radurizing) doses on the crystalline to amorphous ratio and on the permeability of various polymer membranes to gases and water vapour was studied. The average values ($\bar{x}$) and

standard deviations ($\pm s$) of the permeability constants and of the ratio of the crystalline part are presented in the form of column diagrams, where the height of the columns represents the average values and the length of the vertical bar twice the standard deviation.

2.1. Permeability to gases and water vapour

The permeability to gases and water vapour was determined after treatment with food-pasteurizing doses of 50, 100, 200, 400 and 800 krad at 30 °C and characterized by the permeability constant (P) which is expressed in $cm^2\ sec^{-1}\ torr^{-1}$ units. Untreated polymer films tested under identical conditions served as controls.

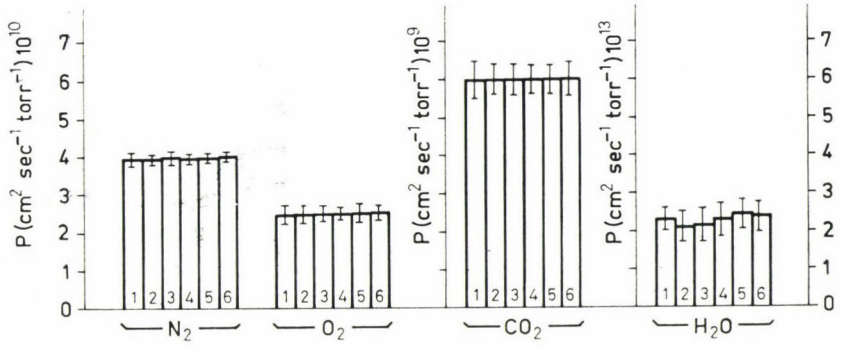

Fig. 4. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0040 cm thick low density polyethylene membrane after treatment with radurizing gamma-radiation to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 28 days elapsed between irradiation and measurement. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *5* — 400 krad; *6* — 800 krad

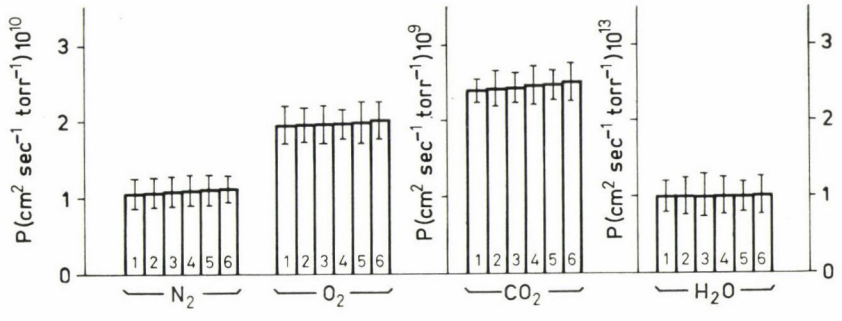

Fig. 5. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0038 cm thick high density polyethylene membrane after treatment with radurizing gamma-radiation to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 35 days elapsed between irradiation and measurement. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

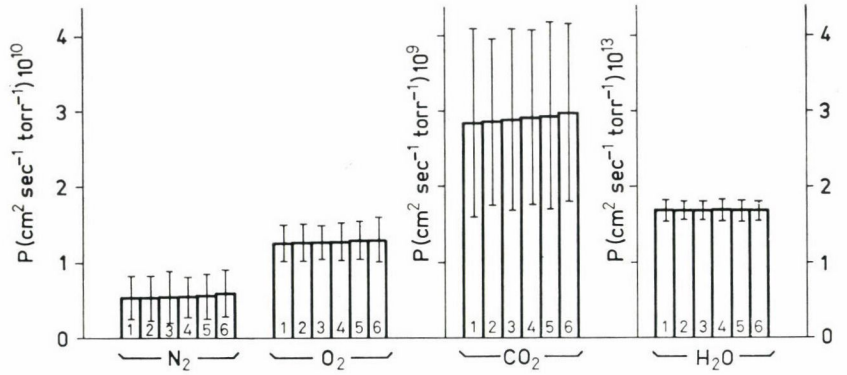

Fig. 6. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0040 cm thick polypropylene membrane after treatment with radurizing gamma-radiation to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 33 days elapsed between irradiation and measurement. Columns: *1* — 0 krad *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

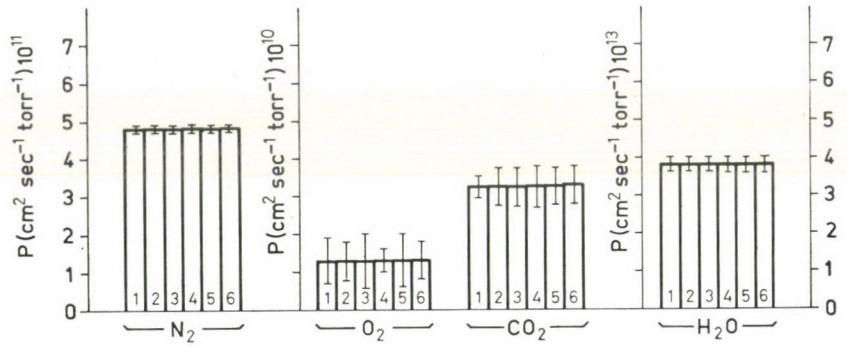

Fig. 7. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0030 cm thick polyvinylchloride membrane after irradiation with radurizing gamma-rays to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 34 days elapsed between irradiation and measurement. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

The results of the determination of the permeability of low density polyethylene to nitrogen, oxygen, carbon dioxide and water vapour is illustrated in Fig. 4.

The measurements were performed 1 to 28 days after irradiation.

The values of the permeability constants of high density polyethylene to nitrogen, oxygen, carbon dioxide and water vapour are shown in Fig. 5.

The measurements were performed during 35 days after irradiation.

The results of the determination of the permeability of polypropylene to nitrogen, oxygen, carbon dioxide and water vapour are illustrated in Fig. 6.

The measurements were performed 1 to 33 days after irradiation.

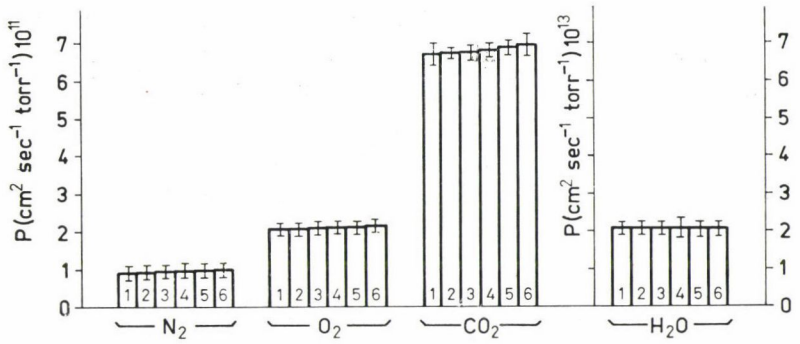

Fig. 8. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0030 cm thick polyvinylidene chloride membrane after treatment with radurizing gamma-radiation to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 34 days elapsed between irradiation and measurement. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

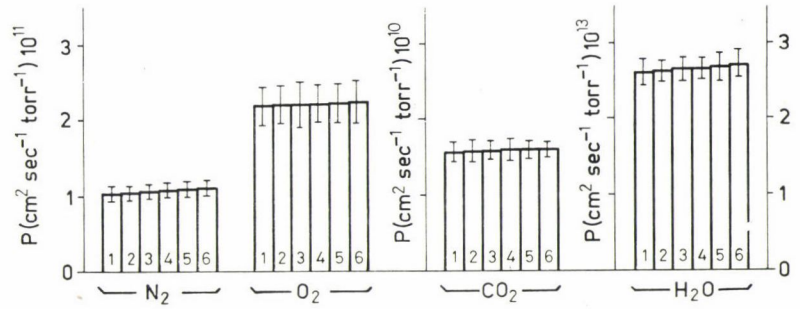

Fig. 9. Comparison of the average values and standard deviations (I) of the permeability constants of a 0.0020 cm thick polyester film after treatment with radurizing gamma-radiation to nitrogen, oxygen, carbon dioxide and water vapour at 30 °C. An interval of 1 to 35 days elapsed between irradiation and measurement. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

In the case of polyvinylchloride the results of the determination of the permeability of the polymer film to nitrogen, oxygen, carbon dioxide and water vapour are shown in Fig. 7.

The measurements were carried out 1 to 34 days after irradiation.

The results of the determination of the permeability of polyvinylidene chloride to nitrogen, oxygen, carbon dioxide and water vapour are illustrated in Fig. 8.

There was an interval of 1 to 31 days between irradiation and the measurement of permeability.

The results of the determination of the permeability of polyester to nitrogen, oxygen, carbon dioxide and water vapour are illustrated in Fig. 9.

The measurements were performed 1 to 35 days after radiation treatment.

It appears from the figures that the differences between the averge values of permeability constants are always smaller than the differences between the average values ± the standard deviations. This finding is supported by the result of two-factor analysis of variance according to which heat treatment caused a very highly significant difference in the permeabilities, while radiation treatment caused no significant difference.

2.2. Proportion of the crystalline part

Changes in the ratio of the ordered, crystalline part to the amorphous part was calculated from the infrared absorption spectra of polymer membranes without radiation treatment and after irradiation with doses of 50, 100, 200, 400 and 800 krad. The results plotted as column diagrams show the appro-

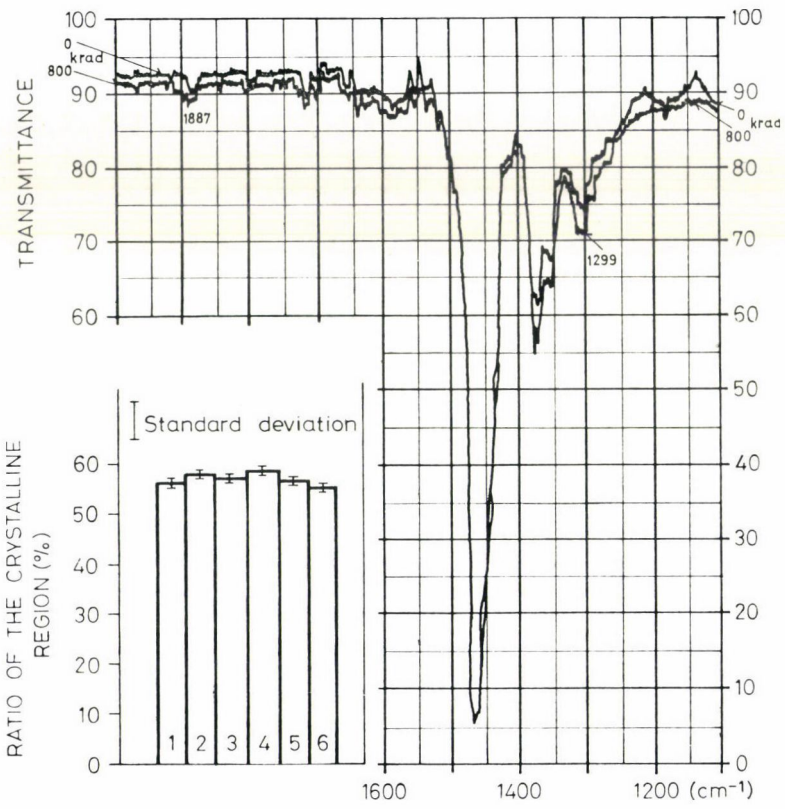

Fig. 10. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0040 cm thick low density polyethylene membranes and comparison of the ratio of the crystalline region in membranes treated with various doses as a function of dose level. Photometry performed immediately after irradiation. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

priate sections of the spectra of the untreated samples and of samples which have been treated with 800 krad. Photometry immediately followed irradiation.

The results obtained for low density polyethylene are shown in Fig. 10, those for high density polyethylene in Fig. 11, for polypropylene in Fig. 12, for

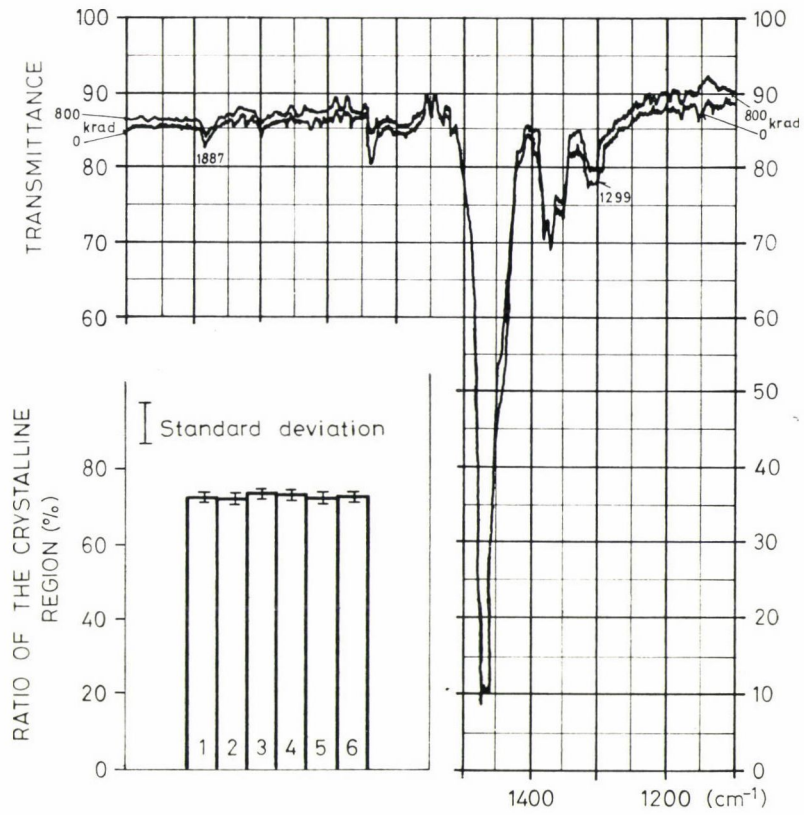

Fig. 11. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0038 cm thick high density polyethylene membranes and comparison of the ratio of crystalline region in membranes treated with various doses as a function of dose level. Photometry performed immediately after irradiation. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

polyvinylchloride in Fig. 13, for polyvinylidene chloride in Fig. 14 and for polyester in Fig. 15.

It appears quite clearly from the figures that irradiation caused a slight, and by no means unidirectional change in the crystalline proportion of the polymers. The column diagrams show further that the differences between the average values (expressed in per cent) of the crystalline parts $\pm$ the standard deviations are always smaller than the differences between the average values.

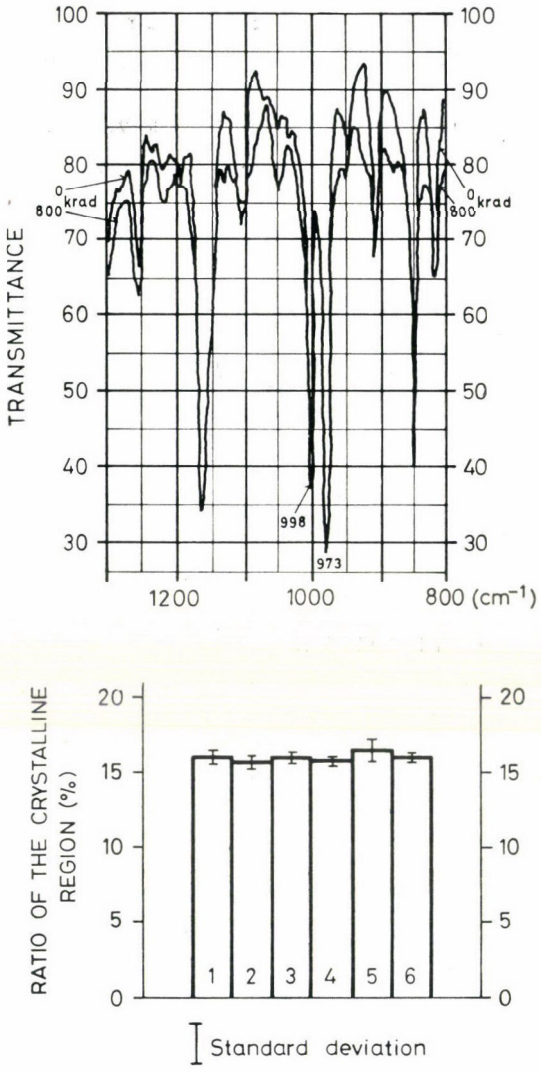

Fig. 12. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0040 cm thick polypropylene membranes and comparison of the ratio of the crystalline region in membranes treated with various doses as a function of dose level. Photometry performed immediately after irradiation. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

The results of one-factor analysis of variance were essentially in agreement with the above, since in none of the cases could a significant difference be detected between the proportions of the crystalline parts as a function of radiation treatment.

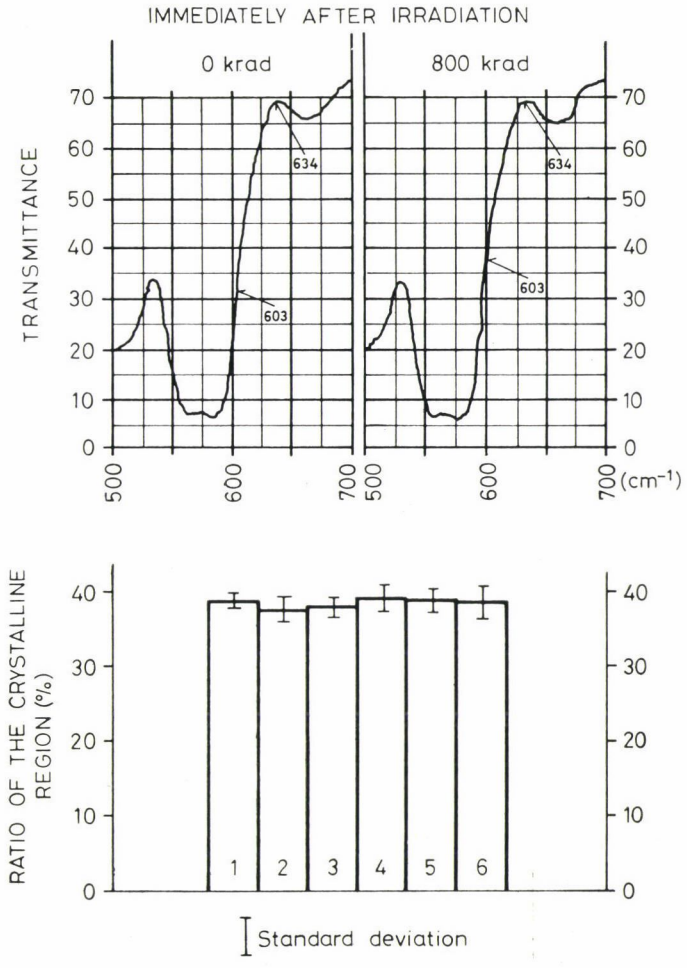

Fig. 13. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0030 cm thick polyvinylchloride membranes and comparison of the ratio of the crystalline region in membranes treated with various doses as a function of dose level. Photometry performed immediately after irradiation. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

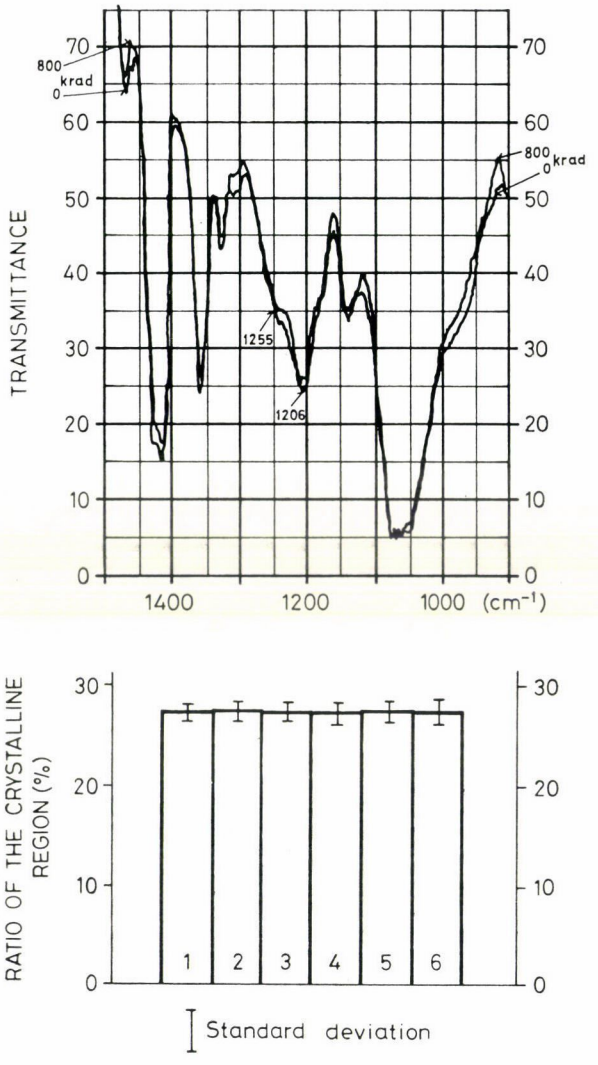

Fig. 14. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0030 cm thick polyvinylidene chloride membranes and comparison of the ratio of the crystalline region in membranes treated with various doses as a function of dose level. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

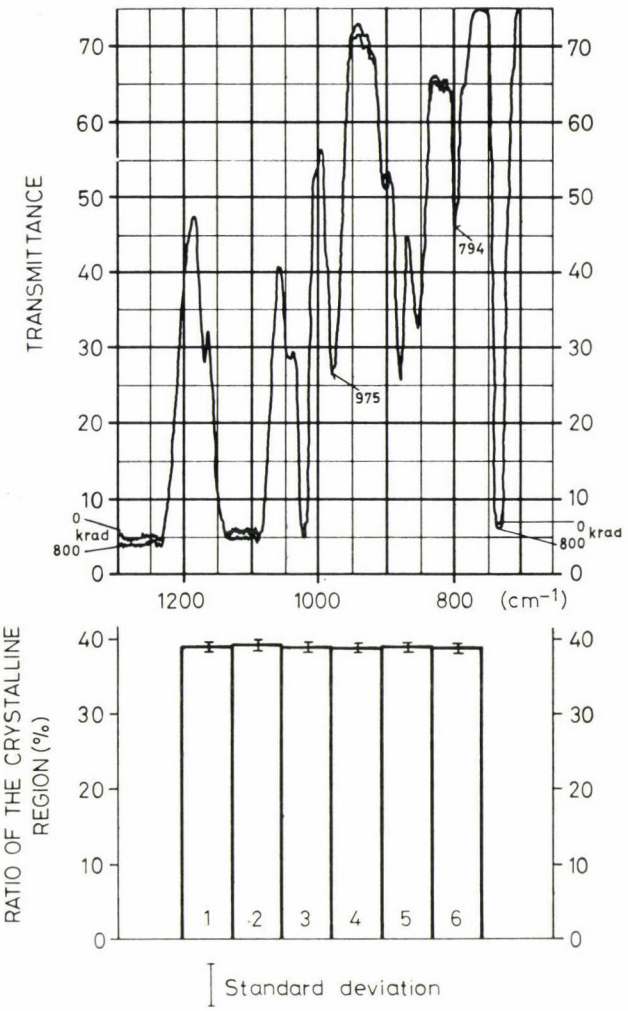

Fig. 15. Infrared absorption spectra of untreated and irradiated (800 krad) 0.0020 cm thick polyester membranes and comparison of the ratio of the crystalline region in the membranes irradiated with various doses as a function of dose level. Photometry performed immediately after irradiation. Columns: *1* — 0 krad; *2* — 50 krad; *3* — 100 krad; *4* — 200 krad; *5* — 400 krad; *6* — 800 krad

3. Conclusions

The results of measurements intended to disclose the effect of radiation treatment on permeability are summed up in Figs. 4 to 9. It appears clearly from the figures and from the results of analysis of variance that doses of 800 krad cause no significant difference in permeability compared to the permeability of untreated polymer films, at the same temperature and to the same permeating substance.

It follows from these observations that doses smaller than 800 krad will cause no changes which might result in significant differences of the sorption or diffusion parameters of, that is in the mechanism of permeability through the investigated polymers. This in fact means that doses of radurizing effect do not represent energies which might cause a change in the number of active sites from the aspect of adsorption, or an excited state which might influence the free route within the polymer, that is the extent of active volume from the point of view of permeability.

Beyond these theoretical considerations another conclusion can also be drawn: namely that for membranes treated with ionizing gamma-radiation the features for the permeability of untreated membranes are valid without correction. This is a very favourable finding from the practical point of view.

The results of the determination of the ordered crystalline part in polymers as a function of radiation treatment unequivocally support the above findings. As illustrated in Figs. 10 to 15 radiation treatment causes no significant change in the crystalline part of polymers, compared to that of untreated polymers. Thus ionizing gamma-radiation of 800 krad causes or initiates no change in the structure of polymers which could be detected by infrared photometry.

This finding is of particular importance as far as the food industry is concerned, since it means that doses even as high as 800 krad will not induce changes in the investigated polymers which might influence unfavourably their hygienic properties. Thus, these polymers can be treated with radurizing doses even when in direct contact with the foodstuff.

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Acta Alimentaria, Vol. 4 (3), pp. 271—289 (1975)

DETERMINATION OF PECTINMETHYLESTERASE, POLYGALACTURONASE AND PECTIC SUBSTANCES IN SOME FRUITS AND VEGETABLES

PART I. STUDY INTO THE PECTOLYTIC ENZYME CONTENT OF TOMATOES

K. Pozsár-Hajnal and M. Polacsek-Rácz

(Received June 13, 1974)

After investigating the conditions of enzyme extraction from the tomato tissues, the pH optimum of pectinmethylesterase (PME) was found to be pH 7.5, while that of polygalacturonase (PG) was 4.5.

To determine the PG activity incubation at 50 °C proved to be most suitable. The PME activity was measured at 30 °C, though the activity was established at 50 °C was about 30% higher.

Pectins esterified to a higher degree form a better substrate for PME activity measurement, while for the assessment of PG activity pectins of lower esterification are more suitable.

The activity was studied as a function of reaction time and enzyme concentration. As seen from the results, in the first stage of the reaction, PME activity was in direct proportion with both reaction time and enzyme concentration. The reduction of viscosity and degree of breakdown as caused by PG activity showed a linear correlation with the square root of reaction time at an enzyme-substrate ratio suitable from the measuring technical aspect for the reaction to take place. At the same reaction mixture composition, activity and enzyme concentration are in linear correlation in a wide range.

The pectolytic enzyme activity was studied in fresh fruits, in fruit frozen in the laboratory, in samples commercially quick-frozen and at various stages of ripening. The PG activity was found to decrease during frozen storage, while the PME activity remained almost unchanged. However, upon freezing and thawing repeatedly the activity of both enzymes was reduced. Both the PG and PME activity increased during ripening in the fruit tissue. When tomatoes were exposed to after-ripening in the refrigerator the PME activity was found to decrease, while the PG activity increased.

Texture change (softening) taking place in fruits and vegetables during ripening and storage is probably due mainly to the enzymic degradation of pectin, responsible for the firmness of plant tissues. This process may become undesirable during storage (Vas, 1954). There are a great number of ways of breakdown, dependent on the kind of product and on the pectin and pectolytic enzyme present in the tissues of the plant under investigation. Thus it is very important to study the pectin and pectolytic enzyme components of the individual fruits and vegetables (Doesburg, 1965; Gizis, 1964).

The knowledge of the pectin and the pectolytic enzyme components of tissue of a certain produce may help in the selection of varieties of longer storage life, or, by the appropriate adjustment of storage conditions or by inhibition of enzymes, to extend their storage life.

Pectolysis plays an important part in fruit juice production, too, by way

of increasing the juice yield (TÖRÖK *et al.*, 1953) and of clarification (VAS, 1953). Several enzyme components take part in the breakdown of pectin, which are present simultaneously and therefore their activity is difficult to measure individually. Two enzyme components were investigated in this study.

Pectinmethylesterase (PME) hydrolyzes the methylester bonds of the pectin chain built from methylated galacturonic acids. In this reaction carboxyl groups are liberated beside the formation of methyl alcohol. The quantity of the carboxyl groups thus liberated may be determined by titration with alkali (KERTESZ, 1955; KOVÁCS-PROSZT, 1965—1966). Other methods are based on determining the methyl alcohol liberated. In this study the former method was used.

Polygalacturonase (PG) is a depolymerizing enzyme and degrades by hydrolysis the glucoside bonds of the main chain of polygalacturonic acid. In plant tissues, however, a precondition of its activity is the demethylating activity of PME, since the completely methylated pectin cannot be attacked by PG. The PG activity is not affected by the breakdown products, but a lower activity is observed in relation to shorter chains. The first step during pectolysis is the reduction of viscosity in the reaction mixture, next the cloudiness decreases and finally the reducing capacity increases. The latter two changes appear only after a significant drop in viscosity. The increase in reducing capacity is difficult to follow in fruit extracts because there are many other reducing substances present in the juice. Thus the PG activity of the various fruit extracts was characterized by their viscosity reducing capacity (VAS, 1953; ZETELAKI-HORVÁTH, 1972).

Of the fruits studied the highest pectolytic activity was found in tomatoes. Therefore tomatoes were used to investigate the properties of plant tissue pectinase (temperature dependence, pH optimum, kinetic studies). The results obtained were utilized in relation to other fruits, though it is not sure that the parameters found optimal in the case of tomatoes, are optimal with the enzymes of other fruits. However, in the other fruits tested these enzymes occur only in lower quantity, sometimes just detectable, therefore those are not suitable for detailed examination. In addition, since only a small amount of tomato was needed in the substrate, the substrate present in the extract beside the enzyme did not affect the result. On the other hand in other fruits, where the enzyme containing extract in relation to the substrate is high, the pectin content in the extract may cause trouble.

In the first part of this series an account is given of the pectolytic enzymes in the tomato tissue.

1. Materials and methods

1.1. The fruit tested

The tomatoes used in the investigations were bought on the market. They were tested fresh, after storage in the refrigerator at +5 °C and —20 °C, resp. In certain cases commercially frozen tomatoes (MIRELITE FACTORY, Budapest) were also used.

1.2. Enzyme extraction from the plant tissue

In the study of tissue enzymes one of the most important tasks is to liberate the enzyme from the plant tissues. Many different methods of extraction were applied by a great number of authors (KOVÁCS-PROSZT, 1965—1966; PATEL & PHAFF, 1960a; VAS *et al.*, 1967). The following method was applied by the present authors: distilled water or 5% NaCl solution ("brine"), or 1/15 *M Sörensen* phosphate buffer of pH 7.5 containing 5% NaCl was added in the proportion of 1 : 1 to the comminuted fruit. A suspension was made of the mixture in a blade homogeniser. The homogenate was kept in the refrigerator overnight, then filtered through a cheese-cloth and centrifuged in a refrigerated centrifuge. The supernatant was used in the experiments. The effect of the various enzyme components was studied in identical extracts.

In certain cases the sediment obtained upon centrifuging was mixed 1 : 1 with a suitable solvent (brine, buffer containing salt) kept for an hour at room temperature and filtered through a cheese-cloth. The sediment was thus washed 1—3 times and the effect of residual activity was tested.

1.3. Substrates used in activity measurement

To measure the PME activity, a 0.5% solution of *Obipektin* "Violett-band" (OBIPEKTIN A. G., Bischofszell, Switzerland, apple pectin of 30—36% esterification), or a 0.5% *Pomosin* solution (POMOSIN-WERKE, GMBH, FRG, apple pectin of about 70% esterification) were used. The pH of the substrate was adjusted with NaOH to pH 7.5 prior to measurement. Hereinafter, the *Obipektin* preparation will be called *low-ester pectin* (O), while the *Pomosin* preparation *high-ester pectin* (P).

To measure the PG activity 0.5% low-ester pectin, dissolved in distilled water, or *McIlvaine* buffer of pH 4.5, was used. To find the most suitable substrate solutions of high-ester pectin were also tested.

1.4. Determination of PME

PME activity was measured by the modified method of *Kertesz* (VAS *et al.*, 1967). Sixty ml were taken of the 0.5% pectin solution (pH 7.5) as de-

scribed in para. 1.3, an adequate amount of fruit extract was added, the pH was rapidly readjusted and the mixture titrated with 0.1 *N* NaOH for 10 min. During titration the pH was kept at a constant level. The amount of alkali used was recorded every min. The reaction was carried out at 30 °C in a thermostat. Titration was carried out with the impulse titrator, Type OP 504 (Fővárosi Finommechanikai Vállalat, Budapest). The quantity of fruit extract was chosen to use possibly about 0.5 ml min^{-1} alkali solution. With tomatoes, the sample used was 5 ml (or less, made up with the extraction solution to 5 ml).

When the pH-optimum of the enzyme was investigated, both the substrate and the reaction mixture were adjusted to the required pH and kept at it during the reaction.

To establish the temperature optimum, identical volumes of the fruit extract were used as for the investigation of the pH optimum. The substrate and the fruit extract were adjusted to the optimum pH and tested at reaction temperatures 30, 40 and 50 °C, respectively. Activity or enzyme concentration in the fruit extract is calculated as follows:

$$PME\ (\mathrm{U\ ml^{-1}}) = \frac{100 \cdot a}{e \cdot t}$$

where a = 0.1 *N* NaOH used, ml,
e = amount of fruit extract, ml,
t = reaction time, min.

In the knowledge of the enzyme concentration in the extract the enzyme concentration in 1 g fruit or vegetable was calculated.

1.5. Determination of PG

To determine PG activity in fruit extracts of high activity a continuous viscometric method was developed. To 10 ml of the 0.5% low-ester pectin solution (pH 4.5), depending on the enzyme activity, 0.4—0.6 ml appropriately diluted tomato extract was added after preheating. The mixture was blended by air injection. Degradation was carried out at 50 °C and the reduction of viscosity was measured during the first 10—20 min of the reaction at the same temperature with the *Ostwald* viscometer, modified according to *Hungarian Standard* 3256 (MNOSZ 3256). For control the same amount of tomato extract, only boiled, was used with the substrate.

The degree of degradation (D) was calculated on the basis of the water delivery time (6—7 sec), and the time of delivery of the blank (24—26 sec) and the enzyme-containing samples. D is the percentage decrease in specific viscosity as related to the viscosity of the blank and is cal-

culated on the basis of the following equations:

$$\text{Relative viscosity: } \eta_{rel} = \frac{t_{sample}}{t_{water}}$$

$$\text{Specific viscosity: } \eta_{sp} = \eta_{rel} - 1$$

$$D = \frac{\eta_{spC} - \eta_{spS}}{\eta_{spC}} \cdot 100 = 100 - \frac{100\,\eta_{spS}}{\eta_{spC}}$$

where t = time of delivery (sec),
η_{spC} = specific viscosity of the blank,
η_{spS} = specific viscosity of the sample.

Activity was characterized by the 50% specific pectolytic activity (SPA_{50}), understood to mean the number of ml of 0.5% pectin solution degraded in 10 min to 50% by the extract obtained from 1 g tomato. The activity was not characterized by degradation per min, because in case of enzyme concentrations, where viscosity degradation could easily be followed (when 50% degradation was reached in 5—15 min), D was in linear correlation with the square root of reaction time and not with the reaction time itself. Therefore, the concentration of the tomato extract was adjusted in relation to the given amount of substrate so as to obtain unit activity in measurements at 1-min intervals that is to reach 50% D in 10 min. In the knowledge of the enzyme concentration required the enzyme content of 1 g tomatoes of various maturity and differently treated may be calculated by linear interpolation.

The standard deviation of the described method of PG determination, measured in 6 replicates ($n = 6$), and expressed as percentage of the mean, was $\pm 4.5\%$.

2. Results

2.1. Enzyme extraction

Enzyme extraction was carried out with distilled water or NaCl solution, or a buffer solution containing 5% NaCl. For the sake of comparison the extracts were prepared from the same tomato batches and to test their efficiency the enzyme concentrations were related to values obtained in brine. Results are given in Table 1.

As seen in the table the PME activity obtained in the salt-containing buffer solution was 1.63 ± 0.56 times that obtained in brine, while the PG activity was 1.26 ± 0.15 times higher. The PG activity in brine was 5—7 times that obtained in distilled water. A final salt concentration above 2.5% seemed to reduce PG activity.

We have examined on five occasions even that in what degree the total activity measurable in the brine-homogenized pulp could be obtained for the extract. We could only carry out these experiments on the PME en-

zyme component, as measuring PG activity is disturbed by the fibre part of tomato.

The results indicated that on average 66 % ($s = \pm 36$) of the PME activity being measurable in 1 g pulp could be obtained for the liquid.

The bond between the fruit tissue and the enzyme in brine and in salt-containing buffer solution was studied by repeating the extraction three times. The activities of the individual extracts expressed as percentage of the total activity are shown in Fig. 1.

Table 1

*The influence of the solvent upon the extraction of enzyme components**

Enzyme	Extracted in			Standard deviation	No. of extractions
	water	brine	buffer + salt		
PG	15%	100%	126%	±15	9
PME	—	100%	163%	±56	11

* Expressed as percentage of the brine extract

As seen in the figure the first extract contains the major part of the total PME and PG activities, 76 and 81 %, respectively. By washing of the fibres the activity obtained may be increased by about 15—16 %. In subsequent experiments only one extraction was applied.

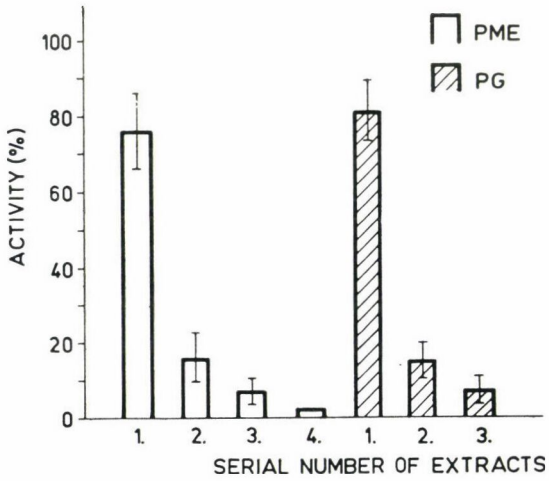

Fig. 1. Extraction of PME and PG enzymes from tomatoes by repeated elution. Substrate: low-ester pectin; solvent: brine and buffer containing salt, extraction made in 3 replicates. Measurement of PME activity: at 30 °C in 6 replicate . Measurement of PG activity at 50 °C in 5 replicates. Data represent the activity of the extracts as percentage of total activity. Vertical bars (I) represent the standard deviations based on 6 or 5 measurements, respectively

2.2. Measurement of PME

2.2.1. PME activity as a function of reaction time and enzyme concentration. In Fig. 2 the alkali required to keep the pH of the reaction mixture at a constant level (pH 7.5) and the carboxyl groups proportionately liberated (in micromole) are shown as a function of the reaction time.

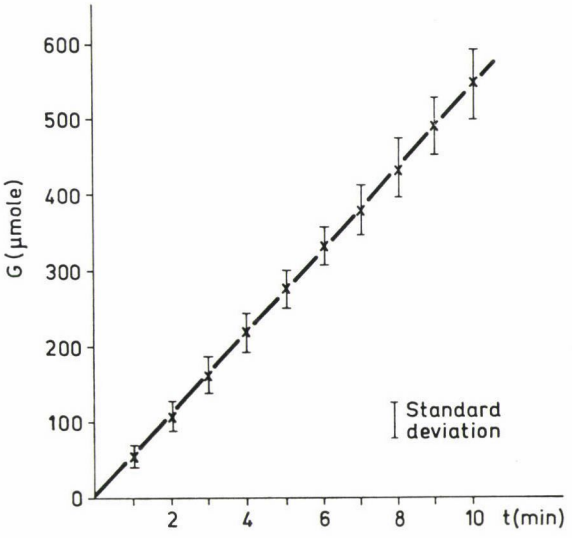

Fig. 2. Liberation of galacturonic acid (G) as a function of reaction time (t). Substrate: high-ester pectin. Enzyme solution: brine extract of tomatoes. Measurement: at 30 °C. Number of parallel measurements = 8. Equation of the regression line: $G = 54.5t + 0.2$; $r = 0.999$

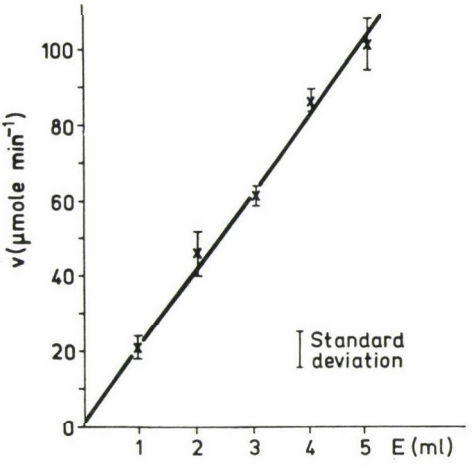

Fig. 3. Correlation between reaction rate (v) and PME enzyme concentration (E = tomato extract in the reaction mixture). Substrate: high-ester pectin. Enzyme solution: tomato extract in buffer containing salt. Measurement at 30 °C. Number of parallels = 4. Equation of the regression line: $v = 20.5E + 1.4$; $r = 0.997$

In the initial phase of the reaction the alkali requirement is steadily increasing, thus, the initial rate of reaction may be characterized by the slope of the linear section. Thus PME enzyme concentration of the fruit extract tested can be calculated by the equation as given in para. 1.4. and can be related to 1 g of fruit.

The correlation between the concentration of the fruit extract and the reaction rate was also linear. This is illustrated in Fig. 3.

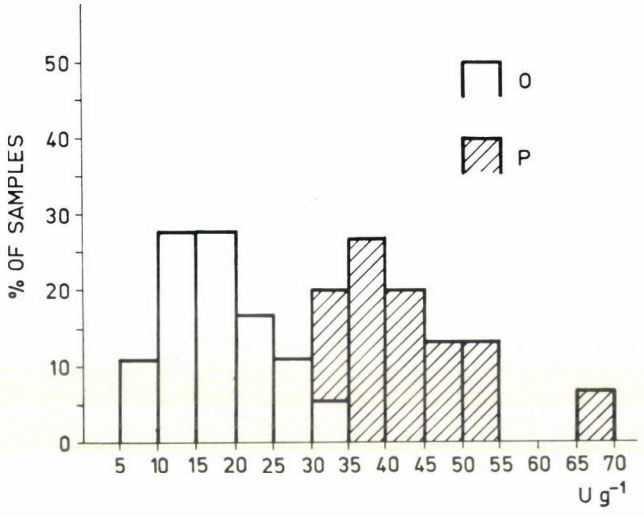

Fig. 4. Frequency distribution of the PME concentration (U g^{-1}) of tomato extracts in brine on low-ester pectin (*O*) and high-ester pectin (*P*), at 30 °C. Number of samples: $O = 18$; $P = 15$

In order to be able to select the substrate of appropriate degree of esterification, the PME activity was determined with both the low-ester and the high-ester pectin. The activity distributions on the 2 substrates are shown in Fig. 4.

As seen, with the low-ester pectin the PME concentration in 72% of the 18 tomato samples was 10—25 U g^{-1}, while with the high-ester pectin 67% of the 15 tomato samples had a PME concentration of 30—45 U g^{-1}. The enzyme concentration as measured with the high-ester pectin was, on the average, 2.44 ($s = \pm 0.8$) times higher than the value obtained with low-ester pectin, thus the former seemed a more suitable substrate to measure PME activity.

2.2.2. The pH optimum of PME. In the case of a brine extract of tomatoes the correlation between PME activity and pH was studied on the two substrates of different degrees of esterification in 3 parallel measurements. The reaction mixture was of the same composition every time. The measurements

were carried out in the pH range 4.0—8.5 at pH 0.5 intervals. The reaction rate as a function of pH is shown in Fig. 5.

As seen, on the low-ester pectin the PME activity is measurable from pH 4.5, while on the high-ester pectin from pH 5.5. The maximum reaction rate was measured on both substrates at pH 7.5, however the values obtained in the range pH 7.0—8.5 did not differ to a significant degree. On the low-ester substrate the reaction rate was lower at pH 7.5, but this did not differ significantly from the values obtained in the range pH 5.0—6.5.

2.2.3. PME activity as affected by temperature. PME activity was measured in the brine extract of tomatoes at temperatures 30, 40 and 50 °C, respectively. The effect of temperature on PME activity is illustrated in Fig. 6.

As seen, in the first 6—8 min of the reaction time the alkali consumption increases steadily, thus the slope of the curve is characteristic of the reaction

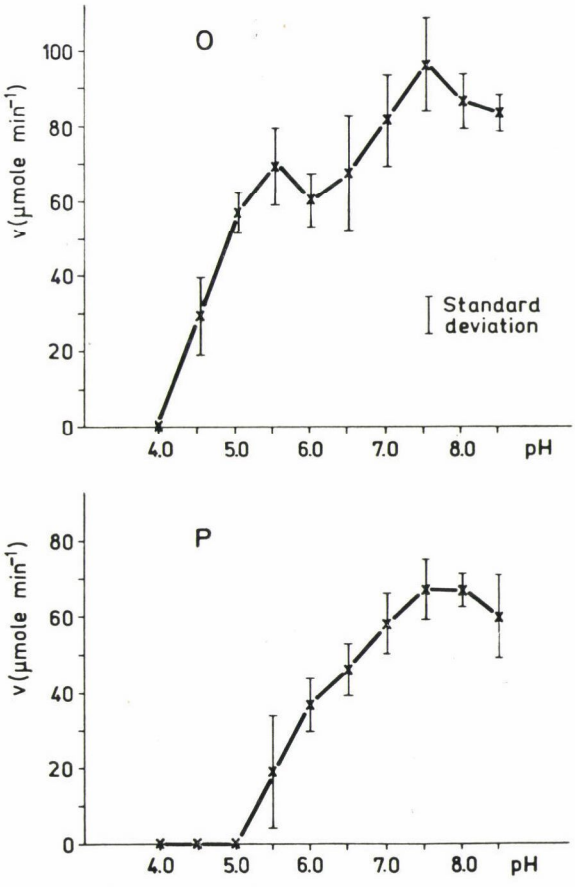

Fig. 5. Study of the pH optimum of the PME activity of tomato extract in brine on low-ester pectin (*O*) and high-ester pectin (*P*), at 30 °C. v = reaction rate; number of parallel measurements = 3

rate. The reaction rate values, as obtained at 30 and 40 °C, do not differ significantly, while those measured at 50 °C were highly significantly higher ($P = 99.9\%$). The *PME* concentration in 1 g tomato was 30% higher at 50 °C than at 30 °C. In spite of this, measurements were carried out at 30 °C, in accordance with the international recommendations.

2.2.4. The effect of maturation and storage on the PME activity of tomatoes. The *PME* activity as measured in tomatoes at various times are given in Table 2.

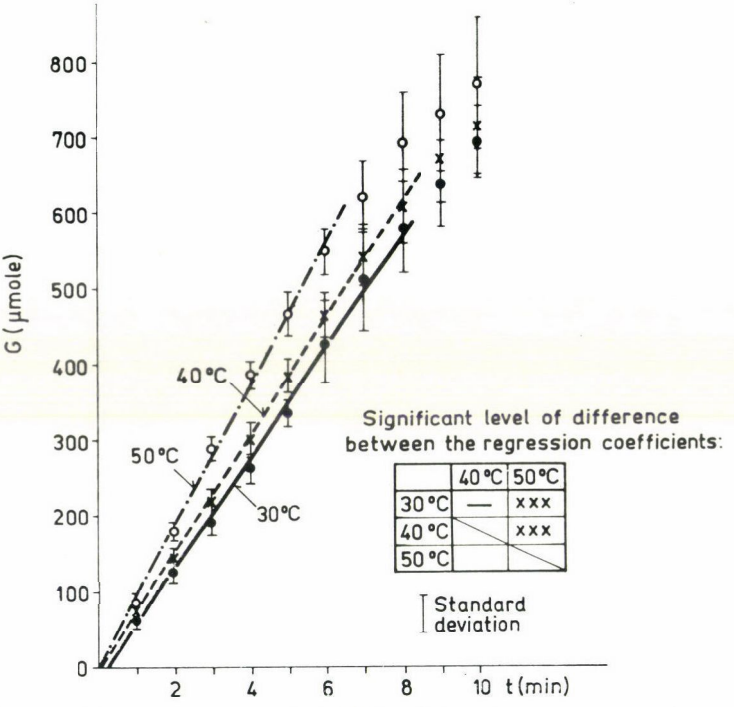

Fig. 6. Influence of temperature on the PME activity of tomato extracts. Liberation of galacturonic acid (G) as a function of reaction time (t). Substrate: high-ester pectin. Enzyme solution: tomato extract in brine. Number of parallels = 3. Equations of the regression lines: at 30 °C $G = 73.4t - 18.0$, $r = 0.998$; at 40 °C $G = 77.0t - 5.7$, $r = 0.999$; at 50 °C $G = 93.1t - 1.3$, $r = 0.999$; $\times\times\times$ = difference between the regression coefficients, significant at the 99.9% probability level; — = difference not significant

The PME activity of tomatoes frozen in the laboratory from the batch studied during 1972, and several times thawed and refrozen (1/f) was about one third of that measured in the fresh sample (1). The activity of half-ripe, commercially quick-frozen tomatoes (2) (MIRELITE FACTORY, Budapest) and of the half-ripe tomatoes grown in hot-house early in 1973 (3) was also low and constituted about 30—50% of that of the samples studied at a riper stage (4, 5). The activity of samples frozen in the laboratory and kept frozen continuously

Table 2

PME and PG activities of various tomato samples in 1 g of fruit, as measured on low-ester substrate

Designation of sample and date of analysis		State of tomato tested	PME activity U g^{-1}	PG activity SPA_{50}
1	1972-10-16	fresh, ripe	23.5	103
1/f	1972-11 – 1973-3	frozen in the laboratory, thawed several times	16.0	60
2	1973-2 – 1973-4	commercially frozen	12.7*	47*
3	1973-6-25	fresh, half-ripe, from hothouse	7.3	58
4	1973-7-4	fresh, ripe	30.2	–
4/a	1973-7-12	stored at +5 °C	17.2	92
5	1973-7-18	fresh, ripe, new sample	21.2	82
5/a	1973-7-23	stored at +5 °C	–	112
6	1973-7-27	fresh, ripe, new sample	16.6	101
6/a	1973-7-30	stored at +5 °C	–	122
7	1973-9-5	fresh, ripe, new sample	27.1	–
7/f	1973-11-8	frozen in the laboratory, stored	26.0	75
8	1973-10-1	overripe, fresh	16.2	128
9	1973-10-8	fresh, ripe, new sample	14.8	114
$9/f_1$	1973-11-8	frozen in the laboratory, stored	14.9	66
$9/f_2$	1974-2-8	frozen in the laboratory, stored	15.4	65
10	1973-10-24	*Lucullus*, fresh	19.0	132

– = not studied
f = a part of the fresh tomato sample of the same number, after several months frozen storage
a = a part of the fresh tomato sample of the same number, kept in the refrigerator for several days
Results marked * are the mean values of 5 parallel measurements, the rest being the result of individual measurements.

for one or several months was also tested and was found to comply with that of the original sample (samples 7 and 7/f; 9, $9/f_1$ and $9/f_2$, resp.). If, however, the tomatoes were kept in the refrigerator for 1 week without being frozen (at +5 °C), the *PME* activity decreased by about 45% (samples 4 and 4/a).

2.3. Measurement of PG activity

2.3.1. PG activity as affected by temperature and the substrate. The influence of the temperature and of the quality of the substrate upon PG activity in the brine extract of tomatoes was investigated. The extract was mixed with the

0.5% solution of the low-ester or the high-ester pectin and incubated at 40 °C or 50 °C, respectively. The results are shown in Fig. 7.

As seen in the figure, the low-ester pectin is a better substrate for the PG enzyme than the high-ester pectin. At 50 °C a higher PG activity was measured than at 40 °C. Therefore in further experiments PG activity measurements were carried out at 50 °C on the low-ester pectin as substrate.

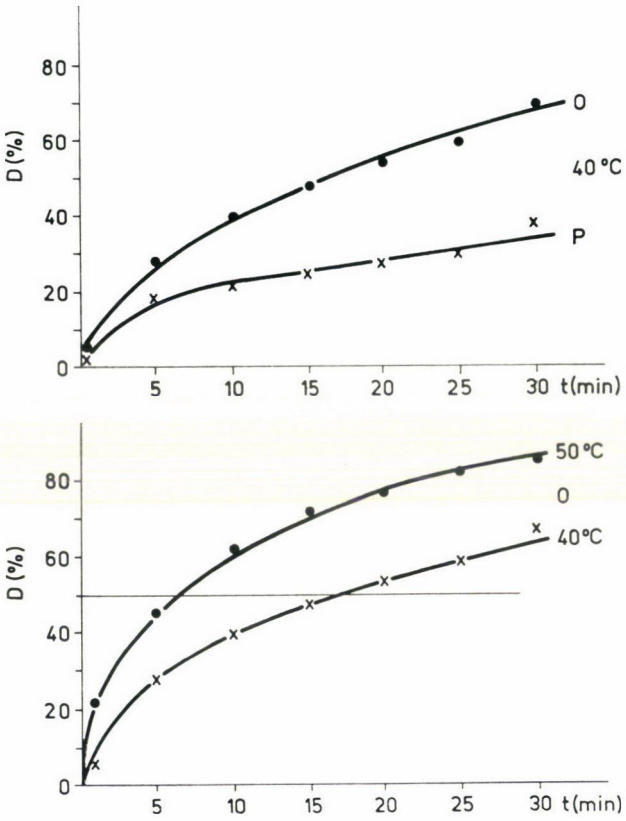

Fig. 7. Measurement of the PG activity of tomato extract on low-ester pectin (*O*) and on high-ester pectin (*P*) at 40 and 50 °C, resp. Degree of breakdown (*D*) as a function of reaction time (*t*). Substrate: 10 ml of a 0.5% pectin solution of pH 4.5. Enzyme solution: obtained of commercially frozen tomatoes (marked 2, see Table 2), 1 ml brine-extract, diluted 4-fold

In the figures illustrating PG activity the deviation of results is not shown, because, due to the accuracy of the method, when measurements were repeated from the same solution the results were highly similar ($V = \pm 4.5\%$), thus it seemed superfluous to replicate and it would have been impossible to illustrate the slight differences.

Out of the components of the reaction mixture only the substrate may be

preheated to 50 °C, because the enzyme gradually becomes inactivated if the tomato extract is kept at 50 °C for a longer period. This is shown in Fig. 8 on a tomato extract kept for various periods at 50 °C.

Thus the tomato extract was preheated only to 30 °C for the measurement. This did not cause a substantial error in the activity measurement, since the quantity of the tomato extract is very low in comparison to that of the substrate. It is sufficient to add 0.4—0.6 ml of appropriately diluted tomato extract to 10 ml substrate, thus the initial temperature and thereby the initial rate of reaction does not change substantially.

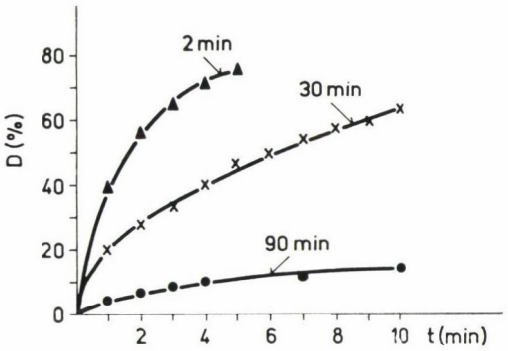

Fig. 8. The effect of the preheating time at 50 °C upon the PG activity of tomato extract. Degree of breakdown (D) as a function of the reaction time (t). Substrate: 10 ml of a low-ester pectin solution of pH 4.5. Enzyme solution: 1 ml tomato extract in brine, diluted 4-fold (sample 1/f, see Table 2). Preheating time: 2, 30 and 90 min, resp. Measurement at 50 °C, every min

2.3.2. PG activity as a function of reaction time. The development of D as a function of reaction time upon the addition of various amounts of tomato extract is shown in Fig. 9.

The figure shows the viscosity-reducing effect of a brine extract of tomatoes on low-ester pectin substrate as a function of time. The standard deviation, expressed as percentage of the average of 3 replicates, was $\pm 3.9\%$. It is apparent that in this concentration range (between 0.4 and 0.6 ml) the reduction of viscosity reaches 50% D in about 5—15 min. Thus it is easy and simple to measure the reduction of viscosity and D is in linear correlation with the square root of reaction time and not with reaction time itself. This correlation is valid up to about 70% degradation and it is not valid for higher enzyme concentrations (0.8—1.0 ml). Therefore that enzyme quantity was considered of unit activity, which was sufficient to reach, under the experimental conditions, 50% D in 10 min. In the knowledge of this it is possible to calculate, by linear interpolation, the specific pectolytic activity related to 1 g tomato and based on this the PG activity of various tomato batches

may be compared. Since the activity of the individual batches depends on their maturity and storage conditions, to obtain unit activity different enzyme concentrations (quantity of extract, dilution) are required. Above 70% D the amount of polygalacturonic acid components of shorter chain

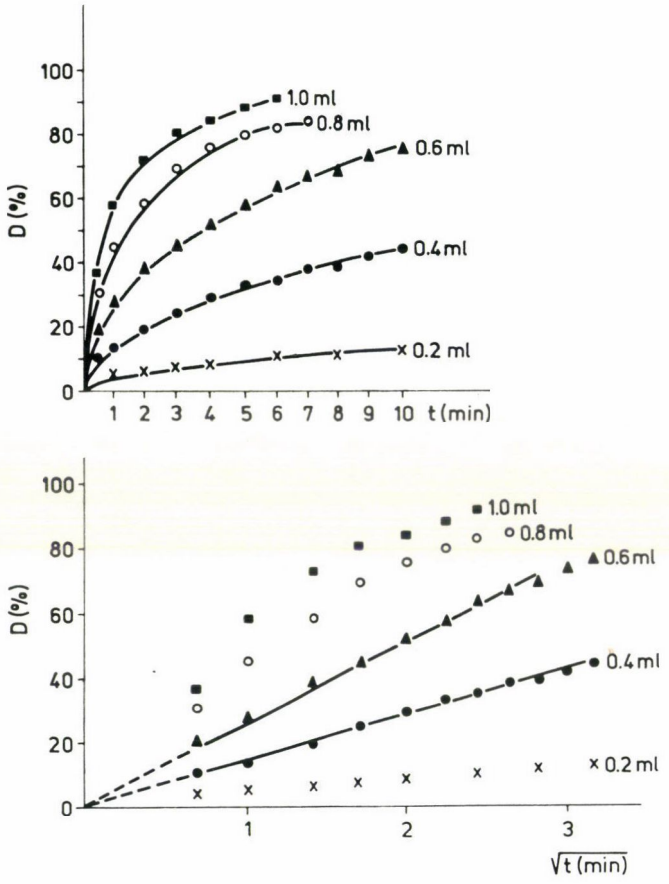

Fig. 9. Correlation between degree of breakdown (D) and reaction time (t) during PG activity measurement. Substrate: 10 ml of a 0.5% low-ester pectin solution of pH 4.5. Enzyme solution: 0.2, 0.4, 0.6, 0.8 and 1.0 ml, respectively, of a tomato extract in brine, diluted 4-fold, prepared of sample 1/f frozen in the laboratory (Table 2). Measurement: at 50 °C during 10 min, every min. Number of parallel measurements = 3. Mean value of the variation coefficient (V) 3.9%. Equations of the regression lines: at 0.4 ml $D = 13.5t + 1.5$, $r = 0.980$; at 0.6 ml $D = 24.5t + 2.1$, $r = 0.970$

increases. The affinity of PG enzyme to these components is lower, therefore, the rate of degradation decreases (VAS, 1953).

2.3.3. PG activity as a function of enzyme concentration. D as a function of enzyme concentration is shown in Fig. 10.

It is apparent that in the range between 15 and 70% D, the latter is in direct proportion to the enzyme concentration needed for the degradation; 50% D, used to calculate unit activity, is in the middle of this range.

2.3.4. PG activity as a function of the pH of the reaction mixture. The correlation between polygalacturonase activity and the pH of the reaction mixture was also studied. The pH of the aqueous solution of the low-ester pectin is 3.6—3.8. This was adjusted to 4.0, 4.2, 4.5, 4.8, 5.0, 5.5 and 6.0, resp., with

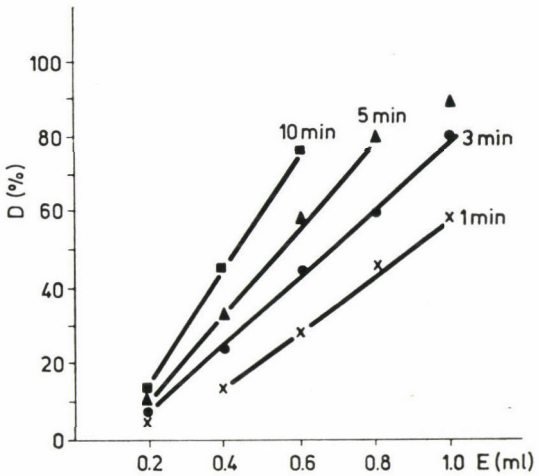

Fig. 10. Degree of breakdown (D) as a function of enzyme concentration (E, ml tomato extract in the reaction mixture) during PG activity measurement. Substrate: 10 ml of a 0.5% low-ester pectin solution of pH 4.5. Enzyme solution: 0.2, 0.4, 0.6, 0.8 and 1.0 ml, respectively, of the brine extract of a tomato sample (Table 2), diluted 4-fold. Measurement: at 50 °C. Reaction times: 1, 3, 5 and 10 min, respectively. Number of parallels = 3. Mean value of the variation coefficient (V): 3.9%. Equations of the regression lines: at 1 min $D = 74.1E - 16.5$, $r = 0.970$; at 3 min $D = 89.0E - 11.2$, $r = 0.960$; at 5 min $D = 113.3E - 12.0$, $r = 0.980$; at 10 min $D = 154.6E - 16.1$, $r = 0.990$

phosphate-citrate buffer. The degree of degradation as a function of pH at various reaction periods is illustrated in Fig. 11.

As seen, the activity rapidly decreases below pH 4.0 and above pH 5.0, while in the range between pH 4.0—5.0 it hardly changes. Thus in the experiments the pH of the substrate was adjusted to pH 4.5.

In the low-ester pectin solution of pH 4.5 the PG activity originating from tomatoes is about 4.5 times higher than in the aqueous substrate of pH 3.6—3.8.

2.3.5. The influence of ripening and storage upon the PG activity of tomatoes. The PG activity of the brine extract of fresh and frozen tomatoes of different maturity and stored in different ways was determined. Results are given in Table 2.

On the basis of the activity data of tomatoes, studied during 1973, it was established that PG activity increased with the advance of ripening. This was apparent in samples harvested at various stages of ripening and the activity was found to increase during after-ripening in the refrigerator at +5 °C. In contrast to this, the PME activity decreased during storage in the refrigerator.

It seems that the PG enzyme is damaged by freezing the tomatoes in the laboratory to —20 °C. The activity of the extracts prepared after thawing of

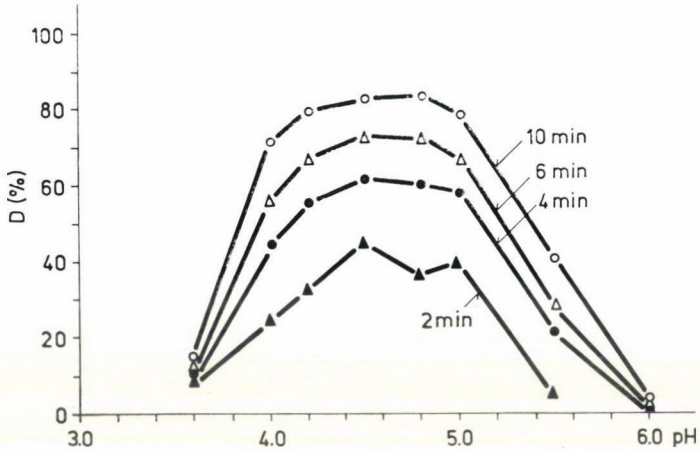

Fig. 11. PG activity (degree of breakdown, *D*) as a function of the pH of the reaction mixture. Substrate: 10 ml of a 0.5% low-ester pectin solution. pH 3.6, 4.0, 4.2, 4.5, 4.8, 5.0, 5.5 and 6.0, resp. Enzyme solution: 0.5 ml of the brine extract of tomato sample 2 (Table 2), undiluted. Reaction time: 2, 4, 6 and 10 min, resp. Measurement: at 50 °C

the samples was about 40% lower than that of the fresh sample (samples 1/f, $9/f_1$ and $9/f_2$). The PME activity of the same samples, however, remained unchanged (samples 7, 7/f; 9, $9/f_1$ and $9/f_2$). Both activities were damaged by repeated thawing and freezing (samples 1 and 1/f).

3. Conclusions

The results of these experiments prove the observations found in the literature (Vas *et al.*, 1967), according to which pectolytic enzymes may be more successfully extracted with a NaCl solution than with water (Table 1). A solution of 2.5% NaCl was found to be the optimum concentration, a higher concentration usually reduced the activity. In preparing tomato extracts a 1/15 *M*, pH 7.5 buffer solution proved even more effective than the salt solution. The buffer extract from 1 g tomato had a PG activity 1.3 times higher, and a PME activity 1.6 times higher than the brine extract.

By washing the homogenate several times the intensity of the adherence of the enzyme to the tissues was tested. For both the PME and the PG, the first extract contained the major part, 76 and 81%, respectively, of the total activity (Fig. 1). Thus the first extract was used to study the tissue enzymes of tomatoes.

This study on PME activity corroborated the observation reported in the literature, namely that the amount of gluconic acid liberated during de-esterification in the initial phase of the reaction was directly proportional both reaction time and enzyme concentration (Figs. 2 and 3) (VAS *et al.*, 1967; NAKAGAWA *et al.*, 1970; KOVÁCS-PROSZT, 1965—1966).

On studying PG activity it was found that with enzyme concentrations suitable to follow the change in viscosity with time, in other words, when 50% D is reached within 5—15 min, D is in direct proportion to the square root of reaction time (Fig. 9). After reaching 70% D, however, presumably due to the accumulation of polygalacturonic acid components of shorter chains, the reaction rate decreases (VAS, 1953).

When PG activity was studied as a function of enzyme concentration, at the same enzyme—substrate ratio, a linear correlation prevailed over an extended range (15—70% D) (Fig. 10). 50% D, used to calculate the activity of the extracts, is almost at the middle of this linear phase.

The pH optimum of PG activity was found, in accordance with literature data (PATEL & PHAFF, 1960b), between pH 4 and 5, thus the pH of the substrate was adjusted to 4.5 (Fig. 11). The pH optimum of PME activity was observed at 7.5 (Fig. 5). Similar observations were made by VAS and co-workers (1967), LEE and MACMILLAN (1968) and NAKAGAWA and co-workers (1970) while studying PME activity in tomato enzyme preparations.

The PME activity of tomatoes is 30% higher at 50 °C than at 30 °C (Fig. 6). This is in accordance with the observation of certain authors (KOVÁCS-PROSZT, 1965—1966) while in contradiction to those of others (VAS *et al.*, 1967). The PG activity was also higher at 50 °C than at 40 °C (Fig. 7).

On studying the quality of the substrate a high-ester pectin was found more suitable for PME activity measurement, while a low-ester pectin proved to be better for the measurement of PG activity. However, both activities could be measured on both substrates (Figs. 4 and 7).

The activity of both enzymes was found to be higher in ripe fruit than in half-ripe one (Table 2). This observation is in accordance with those of authors investigating the change of pectolytic activity in various fruits during ripening (ROUSE *et al.*, 1962, 1964; NAGEL & PATTERSON, 1967; PRESSEY *et al.*, 1971). During after-ripening in the refrigerator the PME activity decreased (samples 4 and 4/a), while PG activity increased (samples 5, 5/a, 6 and 6/a). The activity of the extract did not change during storage in the refrigerator for 1 or 2 days. When frozen under laboratory conditions no change was observed in the PME

activity as compared to that of the fresh fruit, but the PG activity decreased (samples 7 and 9). Both activities were damaged by repeated freezing and thawing (sample 1).

Since these experiments were carried out in a single season on a few samples only, they are of informatory nature only. It would be of interest to perform activity measurements on several samples during several seasons parallel to the study of the pectin content and pectin components of the same samples.

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Thanks are due to E. HEGEDÜS-VÖLGYESI, É. FRIMM-MIZSEI and I. NAGY for their co-operation in the experiments.

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Acta Alimentaria, Vol. 4 (3), pp. 291—308 (1975)

DETERMINATION, LOCALIZATION AND HEAT INACTIVATION OF PEROXIDASE IN SOME VEGETABLES

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(Received September 2, 1974)

A method was adapted from the literature to measure the peroxidase activity of vegetable homogenates. The potato variety "Gül baba" was applied as a model substance throughout the experiments and activities of another 11 products were determined under the appropriate reaction conditions. Activity measurement was based on the spectrophotometric determination of the optical density (at 1 cm path length, *OD*) of the reaction product formed by oxygen liberated from the substrate H_2O_2 upon enzyme action and a chromogenic substance added to the reaction mixture. A linear plot was obtained between *OD* as measured against a blank and reaction time, thus enzyme activity may be characterized by the slope of the regression curve, *i.e.* by the change in *OD* per min. The enzyme is considered to be of unit activity if it brings about a change in *OD* of $1 \cdot 10^{-3}$ per min. On the basis of the relationship between reaction rate and substrate concentration the optimum value of the latter was found to be 0.025% (w/v) H_2O_2 ($7.35 \cdot 10^{-3}$ *M*).

Peroxidase activity of "Gül baba" potatoes shows a maximum at pH 5.0. Enzyme activity increases with temperature in the range from +7 to +55 °C.

Heat resistance and reactivation capacity of peroxidase after heat treatment are different for different products. Peroxidase activity in potatoes is zero after a heat treatment of 10 min at 95 °C, whereas in kohlrabi activity is measurable even after exposures of the same duration to temperatures above 100 °C and reactivation of the enzyme may be observed, too.

The values of peroxidase activity in homogenates of different vegetables were found to differ by orders of magnitude. The accuracy of the method applied is highly satisfactory: coefficients of variation of parallel determinations are below 5% (the number of parallel determinations ranged from 6 to 15).

Peroxidase activity may be detected "*in situ*" in the tissues by the benzidine reaction. Peroxidase activity is higher in the tissues near the skin of the products, distributions in the tissues is homogeneous in black radish and potatoes, whereas the enzyme occurs in zones of higher activity in head of celery.

The enzyme peroxidase has long been known to cause deleterious changes in the colour, taste and flavour substances of vegetables and fruits. The role and properties of the enzyme have, nevertheless, been dealt with by many authors even in recent studies since peroxidase activity is a characteristic biochemical factor of quality not only in vegetable or fruit processing but also in judging cereal grains (GARDNER *et al.*, 1969). Changes in peroxidase activity and the role of the enzyme in flavour formation were studied during ripening of soybeans (RACKIS *et al.*, 1972) and trials were undertaken to find a relationship between the occurrence of the enzyme and lignin formation in pears (RANADIVE & HAARD, 1972). Many research workers were concerned with heat resistance, reactivation capacity and inhibition of the enzyme (EMBS &

MARKAKIS, 1969; ESSELEN & ANDERSON, 1956; MITCHELL & RUTLEDGE, 1973; ROTHE & STÖCKEL, 1967; VOIROL, 1972; WEINRYB, 1966; WINTER, 1968).

However, the methods used to measure enzyme activity are not uniform and investigations into the optimum conditions of measurement are not described in the respective papers.

The investigations published here were aimed at adapting a suitable method from the literature to determine peroxidase activity of vegetables. This involved establishing optimum concentrations of substrate and enzyme for measurement with a given "model" vegetable and applying the method thus developed to study pH and temperature dependence of activity as well as heat resistance and reactivation of the enzyme after heat treatment in some of the products. Further objectives of the work consisted in applying the method to various horticultural products in order to study possible relationships between their respective peroxidase activities and pH values or moisture contents. Finally, beside establishing activity values in vegetable homogenates, the study was completed by detecting the localization of enzyme activity in some of the vegetable tissues by means of a histochemical reaction.

1. Materials and methods

1.1. Materials

1.1.1. The horticultural products. Potatoes of the variety "Gül baba" as purchased in 20-kg lots from primary producers at Budapest markets were used to establish the conditions of measurement of peroxidase activity. Enzyme concentrations are related to wet weight of potatoes with the exception of Table 1 where the respective values are expressed as related to solids content, too.

The method of measurement was applied to determine the respective peroxidase activities of horseradish, cultivated mushrooms, green paprika, head of celery, early and winter kohlrabi, turnips, early and black radish, asparagus and onions. These, too, have been purchased from primary producers and stored at 4 °C until utilization.

1.1.2. The substrate. A fresh substrate solution was prepared daily from 30%, analytical grade hydrogen peroxide (manufactured by REANAL, Budapest) with distilled water.

1.1.3. The o-phenylene diamine solution. A 1% solution of analytical grade o-phenylene diamine (manufactured by REANAL) in 96% ethanol served to indicate peroxidase action.

1.1.4. Buffer solutions. A 0.2 *M* pH 4.0 sodium acetate—acetic acid buffer (18 ml 0.2 *M* sodium acetate + 82 ml 0.2 *M* acetic acid) was used to prepare the homogenates of the vegetables.

The reaction mixtures were adjusted to pH 5.0 (see para. 1.2.1) with a 0.2 *M* pH 5.0 sodium acetate—acetic acid buffer (70 ml 0.2 *M* sodium acetate + 30 ml 0.2 *M* acetic acid).

Disodium hydrogen phosphate—citric acid *(McIlvaine)* buffers in the pH range of 2.4 to 8.1 (DAWSON *et al.*, 1959) were used to study the pH-dependence of peroxidase activity.

1.2. Methods

1.2.1. Preparation of the reaction mixture and determination of enzyme activity. Three pieces each of the products were selected in every series of measurements to prepare the homogenate. These were peeled and finely diced with a stainless steel knife. 10 g of this material were homogenized for 5 min with 90 ml of pH 4.0 buffer in an *Atomix* homogenizer equipped with knives. (The pH 4.0 buffer was applied to inhibit enzyme reaction during comminution.)

Depending on enzyme activity the homogenates of the products were further diluted with the pH 4.0 buffer prior to activity measurement. The final concentrations of the different vegetables as applied in the reaction mixtures are listed in Table 1. One or two ml of the diluted homogenate were pipetted into 50-ml wide-neck conical flasks and made up to 10 ml with pH 5.0 buffer. The blank contained in a total volume of 10 ml only the buffers of pH 4.0 and 5.0 in the same ratio as the reaction mixture.

The flasks were preheated to 25 °C in a 24-place water bath "*Vibrotherm*" manufactured by LABOR MIM, Budapest, and 1 ml each of o-phenylene diamine solution as well as 1 ml each of hydrogen peroxide solution were pipetted both to the blanks and to the solutions containing the enzyme.

After combining the reaction components the mixture was incubated under shaking (aeration of 0.036 mmole O_2 per min) at 25 °C.

After reaction periods of 0, 5, 10, 15, 20 and 25 min, respectively, three flasks each of the reaction mixtures and one flask each of the blanks were removed from the water bath. 2 ml portions of saturated sodium hydrogen sulfite solution were added to each enzyme-containing sample to stop the reaction and the reaction mixtures were made up to 20 ml by adding 6 ml of 96% ethanol. Blanks were treated similarly. After filtration through a folded filter the *OD* as obtained against the blank incubated for the same period were read in a MOM 203 type spectrophotometer at 420 nm. Thus each analysis comprised 1 blank and 3 parallel samples containing the enzyme.

1.2.2. Selection of optimum concentrations of substrate and enzyme, respectively. Analyses were carried out with the following concentrations in order to establish optimum values for substrate and enzyme, resp.:

Concentration of the potato homogenate (%, w/v)	Concentration of the substrate	
	(%, w/v)	$M \cdot 10^{-3}$
0.0166	0.0063	1.83
0.0250	0.0125	3.68
0.0333	0.0188	5.23
0.0415	0.025	7.35
	0.033	9.70
	0.050	14.70

1.2.3. Relationship between peroxidase activity and pH. The effect of pH on the peroxidase activity of potatoes was studied in the pH-range of 2.46 to 8.10 at the following pH-values beside the extreme ones: 3.43, 4.06, 4.76, 5.10, 5.67, 6.60 and 7.05. Enzyme activity was determined as described in para. 1.2.1. pH values of the reaction mixtures were checked in a *Radiometer* PHM 4 pH-meter.

1.2.4. Study of the effect of temperature.

1.2.4.1. Measurement of the peroxidase activity of potatoes as a function of temperature. — The relationship between peroxidase activity and temperature was measured in the range of 7 to 55 °C at 7, 15, 25, 35, 45 and 55 °C, resp. in reaction mixtures of pH 5.0.

1.2.4.2. Study of heat resistance of the enzyme. — After having reached the temperature desired, the potato homogenates were kept for 10 min in a water bath at 65, 75, 85, 90 and 95 °C, resp., in order to establish heat resistance of the enzyme. Following heat treatment residual activities were measured at 25 °C and pH 5.0, according to para. 1.2.1. In this series of experiments the homogenates of the products were prepared with distilled water.

1.2.4.3. Study of the reactivation of the enzyme after heat treatment. — Reactivation of the enzyme peroxidase was studied in homogenates of potatoes and winter kohlrabi, respectively. After heat treatment (10 min at 95 °C for potatoes and equally 10 min at 90, 95, 100, 110 and 120 °C, resp, for kohlrabi) the suspensions were kept at 25 °C for 0, 1 and 2 as well as for 24 h, resp., and residual activities were determined according to para. 1.2.1. Heat treatments at 90, 95 and 100 °C were carried out in a water bath and those at 110 and 120 °C in an autoclave.

1.2.5. Application of the method of measurement to various horticultural products. The homogenates of the products were prepared and activity measurements carried out with all the horticultural products investigated according to para. 1.2.1.

1.2.6. Histochemical studies. Cuttings of black radish, potatoes of the variety "Gül baba" and heads of celery were prepared by means of freezing to demonstrate the enzyme peroxidase in the tissues. Peroxidase was detected by benzidine. The underlying principle of detection is the same as with o-phenylene diamine, namely that, in the presence of an oxygen donor (H_2O_2) as substrate, peroxidase is capable of catalyzing the oxidation of a colourless oxygen acceptor (in the present case benzidine) to a coloured product (benzidine blue). Thus the granuli containing peroxidase are stained blue and then slowly turn to brown. The brown colouration is due to a spontaneous secondary reaction product of benzidine blue (TANKA & KELLER, 1965).

The histochemical reaction was performed in incubating solutions of the pH values of 2.2, 3.6 and 5.0, respectively, at 25 °C with incubation periods of 1 min.

1.2.7. Determination of the solids content of the products. Solids content of the products was assessed by drying to constant weight at 105 °C in an oven.

1.2.8. Determination of the pH of the products. 40—50-g portions of the products were pulped (in some cases with the addition of distilled water) and the pH of the pulp was measured in a pH meter type *Radiometer* PHM 4 (Copenhagen).

1.2.9. Biometrical evaluation of the results. Activity measurements were carried out with 6 to 15 parallel samples. Linear regressions and standard deviations of the regression coefficients were calculated from individual values of *OD* (SVÁB, 1967). Correlation coefficients significant at the probability levels of 99.9 and 99% were marked *** and **, respectively.

2. Results

2.1. Reaction rate as a function of substrate concentration

The relationship between reaction rate (v) as expressed in ΔOD per min and substrate concentration (%, w/v) for reaction mixtures containing 0.0063, 0.0125, 0.0188, 0.025, 0.033 and 0.050% (w/v) hydrogen peroxide, resp., is illustrated in Fig. 1.

According to the results reaction rate has a maximum at a substrate concentration of 0.025% (w/v) which corresponds to 1 ml of 0.3% hydrogen peroxide in the reaction mixture of 12 ml.

The reciprocal plot of reaction rate (1/v) *vs.* substrate concentration (1/S) in the range of substrate concentrations between 0.0063 and 0.025% (w/v) is shown in Fig. 2.

The value of the *Michaelis* constant as calculated from the regression curve is $K_M = 4.37 \cdot 10^{-3}\ M$ and maximum reaction rate was found to be $V_{max} = 8.56 \cdot 10^{-2}\ \Delta OD\ min^{-1}$.

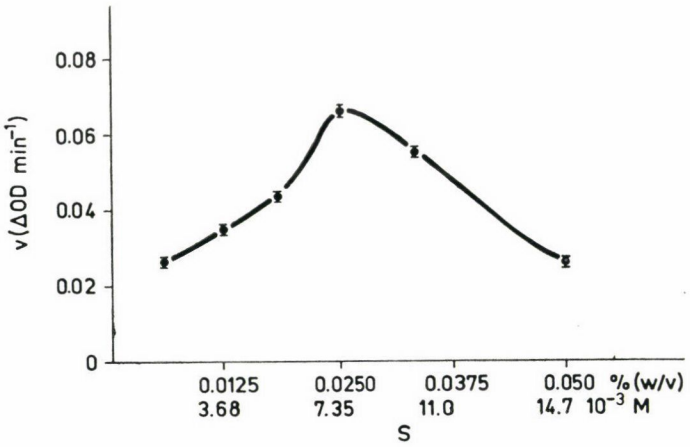

Fig. 1. Relationship between reaction rate and substrate concentration in the case of potato peroxidase. v = reaction rate (ΔOD min^{-1}); S = H_2O_2 concentration % w/v and M, resp.; pH = 5.0; T = 25 °C; potato concentration in the reaction mixture: 0.033% (w/v, wet-weight basis); mean values and standard deviations of reaction rates were calculated from data of 6 parallel samples each. The vertical bars indicate standard deviations ($2s$)

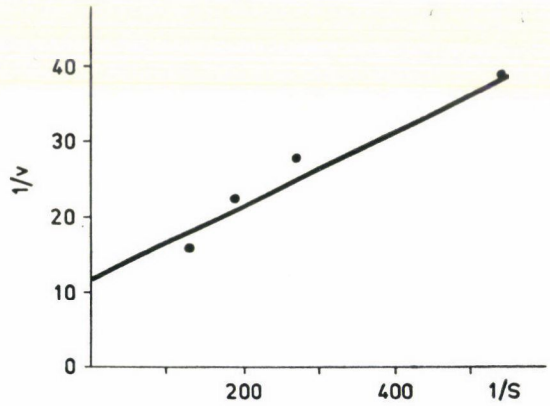

Fig. 2. Relationship between the respective reciprocal values of reaction rate and substrate concentration. (Experimental conditions as in Fig. 1.) v = reaction rate, (ΔOD min^{-1}); S = substrate concentration, M; equation, of the regression curve: $1/v = 0.0512 \cdot 1/S + 11.68$; $r = 0.96^{**}$; $n = 4$; value of the *Michaelis* constant: $K_M = 4.37 \cdot 10^{-3}$ M; $V_{max} = 8.56 \cdot 10^{-2}$ ΔOD min^{-1} (r = regression coefficient; n = number of points of measurement; V_{max} = maximum reaction rate)

2.2. Changes in optical density as a function of reaction time at different concentrations of the potato suspension

The relationship between OD and reaction time (min) as obtained at potato concentrations of 0.0166, 0.0250, 0.0333 and 0.0415% (w/v, wet-weight basis) is presented in Fig. 3.

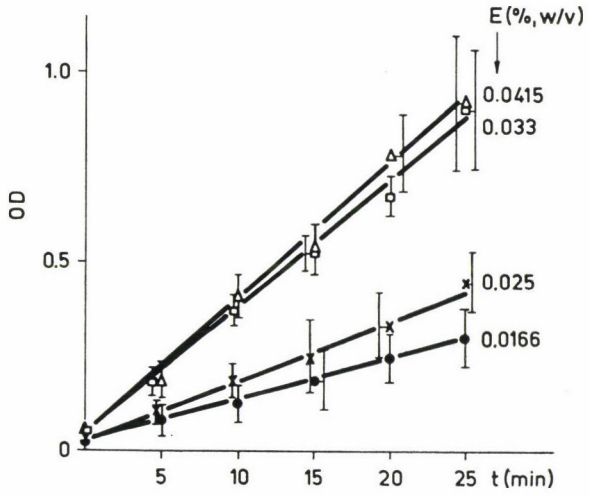

Fig. 3. Relationship between OD and reaction time (t) at various wet-weight potato concentrations (E). Equations of the regression curves at the potato concentration of 0.0166% (w/v, wet-weight): $OD = 0.0105t + 0.026$, $r = 0.78$***, $n = 36$, $r^2 = 0.52$; at the potato concentration of 0.0250% (w/v, wet-weight): $OD = 0.021t + 0.013$, $r = 0.91$***, $n = 36$, $r^2 = 0.83$; at the potato concentration of 0.0333% (w/v, wet-weight): $OD = 0.033t + 0.041$, $r = 0.93$***, $n = 59$, $r^2 = 0.86$; at the potato concentration of 0.0415% (w/v, wet-weight): $OD = 0.036t + 0.035$, $r = 0.92$***, $n = 52$, $r^2 = 0.85$. Regression equations were calculated from 6 to 12 individual values of OD each at 6 reaction periods; pH = 5.0; $T = 25$ °C; $S = 0.025\%$ (w/v). The vertical bars indicate standard deviations ($2s$)

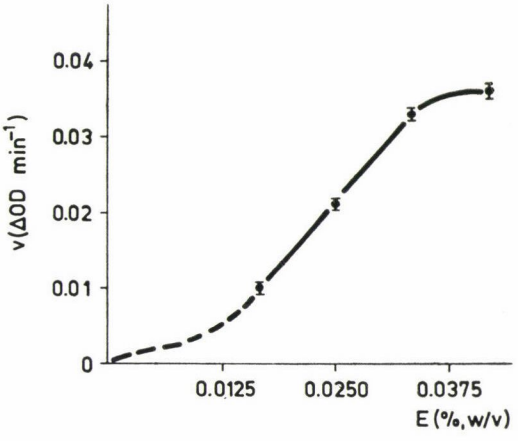

Fig. 4. The reaction rate (v) as measured at different wet-weight potato concentrations (E). pH = 5.0; $T = 25$ °C; $S = 0.025\%$ (w/v); the vertical bars represent the standars deviations ($2s$) as obtained from 6 parallel measurements

Applying the above concentrations the relationship between OD and reaction time is linear in the section of 0 to 25 min of incubation. The correlation was found to be close with potato concentrations of 0.0250, 0.0333 and

0.0415% (w/v, wet-weight basis) ($r = 0.919$, 0.936 and 0.923, respectively), while it was weaker with the sample of 0.0166% (w/v, wet-weight basis) potato content ($r = 0.785$), but was proven highly significant in all the cases.

On plotting reaction rate against potato concentration (Fig. 4), a linear increase in reaction rate is obtained in the range of potato contents between 0.0166 and 0.0333% (w/v, wet-weight basis), a further increase in concentration up to 0.0415% (w/v, wet-weight basis) results, however, in a decrease of the slope of the curve.

2.3. Unit of enzyme activity

Peroxidase activity is defined as the apparent reaction rate (rate of colour change), *i.e.* the change in *OD* per min. The characteristics of the linear section of the plot between *OD* and reaction time (Fig. 3) are calculated by linear regression from individual values of *OD*. The value of enzyme activity is indicated by the slope of the regression curve.

The enzyme is considered to be of unit activity if it brings about a change in *OD* of $1 \cdot 10^{-3}$ per min. Activity values of the products are related to unit wet-weight and will, hereafter, be referred to as enzyme concentration. In the table comparing enzyme concentrations of various vegetables (Table 1) the values are expressed as related to solids content of the product as well.

2.4. Peroxidase activity as a function of pH

Enzyme concentrations of potatoes are shown in Fig. 5.

As can be seen from the figure, enzyme concentration varies with pH according to a maximum curve. At pH 2.46 activity is not measurable, then

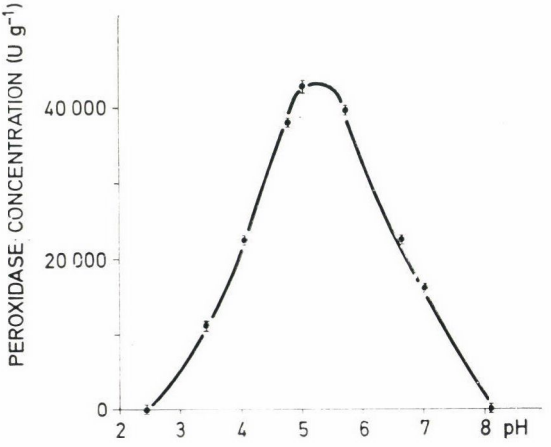

Fig. 5. Peroxidase concentration of the potato variety "Gül baba" as a function of pH. $T = 25$ °C; $S = 0.025\%$ (w/v); potato concentration in the reaction mixture: 0.05% (w/v, wet-weight); the vertical bars represent standard deviations ($2s$) as calculated from data of 6 parallel measurements

a monotonous increase is noted up to about pH 5.0. The curve reaches a maximum between the pH values of 5.0 and 5.4. From pH 5.5 enzyme activity decreases with increasing pH and is below the limit of measurability at pH 8.1.

2.5. Peroxidase activity as a function of temperature

The relationship between peroxidase concentration and temperature in the range of 7 to 55 °C is illustrated in Fig. 6.

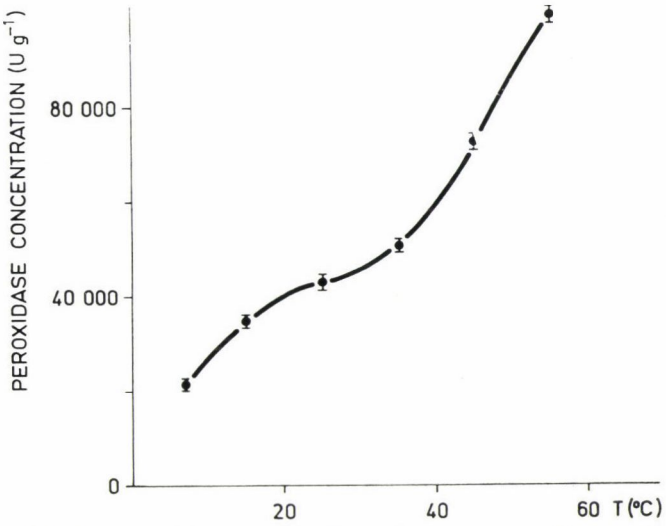

Fig. 6. Relationship between the concentration of potato peroxidase and temperature. pH = 5.0; $S = 0.025\%$ (w/v); potato concentration in the reaction mixture at 7, 15, 25, 35 and 45 °C, resp.: 0.033% (w/v, wet-weight), at 55 °C: 0.0166% (w/v, wet-weight); the vertical bars represent standard deviations (2*s*) as calculated from the data of 6 parallel measurements

The figure shows an increase in activity with temperature over the whole range investigated. The increase in activity is less pronounced in the range between 7 and 35 °C than in the range from 35 to 55 °C. At temperatures above 55 °C uncontrollable processes resulting in brown discolouration take place in the blanks as well as in the samples containing the enzyme and interfere with the determinations.

2.6. Heat resistance of the enzyme

Figure 7 presents residual activities as determined at 25 °C in potato homogenates kept previously at 65, 75, 85, 90 and 95 °C, respectively, for 10 min. Residual activities are expressed as % of the activity of a sample not exposed to heat and are plotted against temperature.

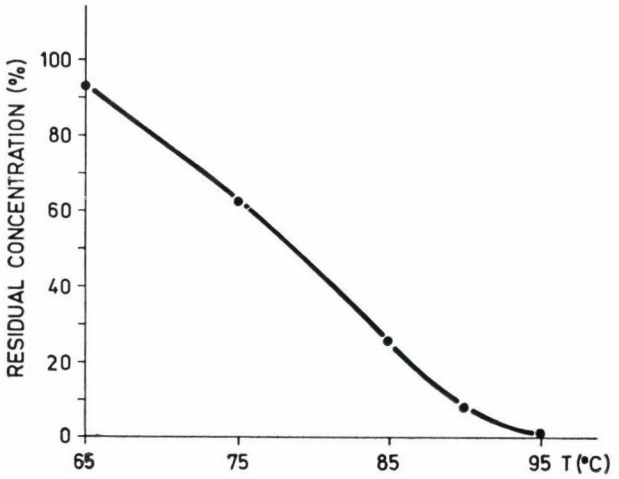

Fig. 7. Residual activities of potato peroxidase as a function of inactivation temperature. Duration of heat exposure: 10 min; $S = 0.025\%$ (w/v); potato concentration in the reaction mixture: 0.033% (w/v, wet-weight); temperature of activity determination: 25 °C; pH = 5.0

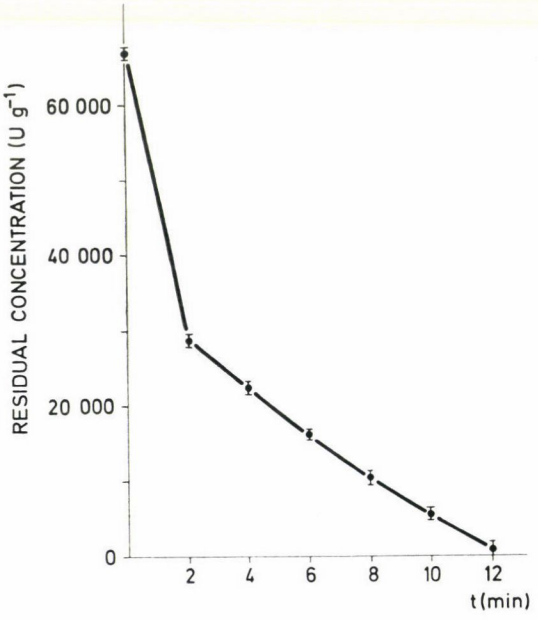

Fig. 8. Residual activities of potato peroxidase as a function of the duration of heat treatment. $S = 0.025\%$ (w/v); potato concentration in the reaction mixture: 0.066% (w/v, wet-weight) at 90 °C. Activity measurements were carried out at 25 °C and pH 5.0. The vertical bars represent standard deviations ($2s$) as calculated from data of 6 parallel measurements

After a 10-min exposure to 65 °C the potato homogenate retains 92.4% of its activity. The respective values for the higher temperatures are: 62.5% for 75 °C, 25.4% for 85 °C and 7.8% for 90 °C. A heat treatment for 10 min at 95 °C results in a virtually complete inactivation of the enzyme.

The relationship between the duration of heat treatment and residual activity was investigated applying exposures to 90 °C. The results are given in Fig. 8.

According to the results the decrease in peroxidase activity of potatoes is abrupt in the first two min and less marked in the following interval up to 12 min.

2.7. Reactivation of the enzyme

In order to study the reactivation of the enzyme residual activities of potatoes exposed for 10 min to 90 °C were determined after storage at 25 °C for 0, 1, 2 and 24 h, respectively. Residual activities did not vary with time. A different behaviour was observed, however, with kohlrabi.

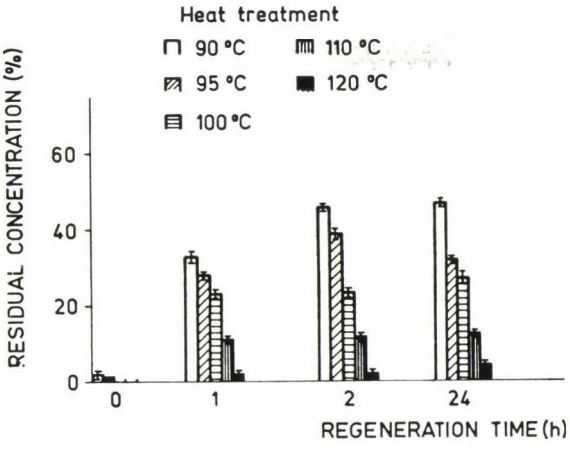

Fig. 9. Reactivation of heat treated kohlrabi peroxidase. Residual activities after different regeneration times. Duration of heat exposure: 10 min; $S = 0.025\%$ (w/v); kohlrabi concentrations in the reaction mixtures: 0.0004% (w/v, wet-weight) with the untreated and 0.0004—0.08% (w/v, wet-weight) with the heat treated samples; activity measurements were carried out at 25 °C and pH 5.0; the vertical bars indicate standard deviations as calculated from the data of 6 parallel measurements

The residual activities of kohlrabi homogenates exposed for 10 min to various temperatures in the range of 90 to 120 °C were determined after storage at 25 °C for 0, 1, 2 and 24 h, respectively. Residual actvities as expressed in percentage of peroxidase activity of an untreated sample are shown in Fig. 9.

According to the results residual activities as determined immediately after heat treatment are below 1.7%. Activity values as measured after identical durations of heat treatment and subsequent periods of waiting decrease with increasing temperature: after 10 min of heat exposure and 60 min of waiting time residual activity was found to be 33.3% in the sample kept at 90 °C, while the respective values for the samples kept at higher temperatures were 29.0 (95 °C), 23.2 (100 °C), 10.7 (110 °C) and 2.1% (120 °C). Reactivation at 25 °C occurs essentially in the first 2 h, the increase in activity as observed between 2 and 24 h did not prove significant.

2.8. Utilization of the method of measurement to determine peroxidase concentrations of some horticultural products

Peroxidase concentrations of various vegetables as determined under optimum conditions of measurement (0.025% w/v substrate concentration, pH 5.0, 25 °C) are summarized in Table 1.

According to experimental data considerably different enzyme concentrations could be established for the various products by the technique developed. The lowest peroxidase concentration (2637 U g^{-1}) was observed in cultivated mushrooms. The values found for onions, turnips, head of celery and potatoes (19 000 — 42 000 U g^{-1}) are higher by one, those of green paprika, early radish, asparagus and early kohlrabi (125 000 to 330 000 U g^{-1}) by two orders of magnitude. Peroxidase concentrations are exceedingly high in black radish, horseradish and winter kohlrabi (1 to 2.5 million U g^{-1}).

2.9. Histochemical investigations

As was to be expected considering the results of quantitative activity determinations, the benzidine test was positive for all the products investigated. In black radish (Fig. 10) and potatoes (Fig. 11) peroxidase is intense and of uniform distribution. However, owing to bad cutting conditions, localization of peroxidase could not be unambiguously established in the cuttings of potatoes. In the cuttings of head of celery (Figs. 12 and 13) the reaction becomes visible more slowly and the enzyme is localized in zones or fascicles. In all three products a higher concentration of the enzyme can be observed near the skin than in the central parts.

Table 1

Peroxidase concentration of horticultural products

Name of the product	Concentration of the product in the reaction mixture (%, w/v, wet-weight basis)	Peroxidase concentration+				Moisture content of the product (%)	pH of the product
		wet-weight basis		dry-weight basis			
		U g^{-1}++	s+++	U g^{-1}++	s+++		
Cultivated mushrooms	2.0833	240	3.3	2 637.3	36.3	90.9	6.41
Onions	0.4166	1 764	54.9	19 173.9	503.4	90.8	5.58
Turnips	0.3333	5 250	182.2	21 255.0	707.6	75.3	6.12
Head of celery	0.1666	3 450	124.8	25 555.5	924.4	86.5	5.90
Potatoes "Gül baba"	0.0333	10 875	263.0	42 647.0	1 031.3	74.5	6.20
Green paprika	0.0208	8 250	230.0	125 000.0	3 484.7	93.4	5.79
Early radish	0.0833	5 360	106.0	130 731.0	2 585.3	95.9	6.20
Asparagus	0.0208	20 480	664.0	305 674.0	9 910.3	93.3	5.80
Early kohlrabi	0.0083	30 400	810.0	330 434.7	8 804.1	90.8	6.25
Winter kohlrabi	0.0033	125 250	4 621.7	1 138 630.0	42 014.3	89.0	6.12
Horseradish	0.0008	568 750	19 370.0	1 687 850.0	57 483.1	66.3	4.90
Black radish	0.0042	102 600	1 467.2	2 565 000.0	36 679.5	96.0	6.32

+ *Conditions of measurement:* Composition of reaction mixture: 1 ml 0.3% H_2O_2, 1 ml 1% o-phenylene diamine, 1 or 2 ml homogenate of product, made up to 12 ml with pH 5.0 buffer. Incubation at 25 °C for 0 – 25 min under aeration, pH of the reaction mixture = 5.0. At the end of incubation addition of 2 ml saturated $NaHSO_3$ and dilution to 20 ml with 96% ethanol. Measurement of OD at 420 nm against a blank treated in the same way and of similar composition, but containing distilled water or pH 4.0 buffer instead of product homogenate. Initial reaction rate is calculated from the linear section of the OD *vs.* reaction period plot by regression analysis, whereby enzyme activity is indicated by the slope of the curve and expressed in ΔOD min^{-1}.

++ The enzyme is considered to be of unit activity (U) if it brings about a change in OD (optical path length: 1 cm) of $1 \cdot 10^{-3}$ per min.

+++ Standard deviations of enzyme concentration values have been established from 6 to 15 parallel measurements and are derived from standard deviations of the regression coefficients. Calculation is based on individual values of OD.

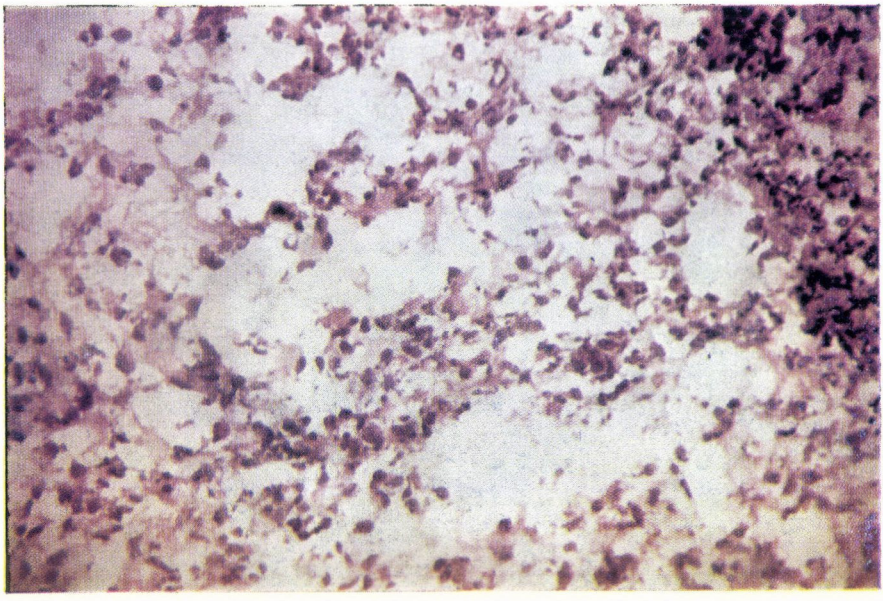

Fig. 10. Peroxidase localization in black radish. pH = 3.6; T = 25 °C; incubation period: 1 min; dye: benzidine; magnified 200 times

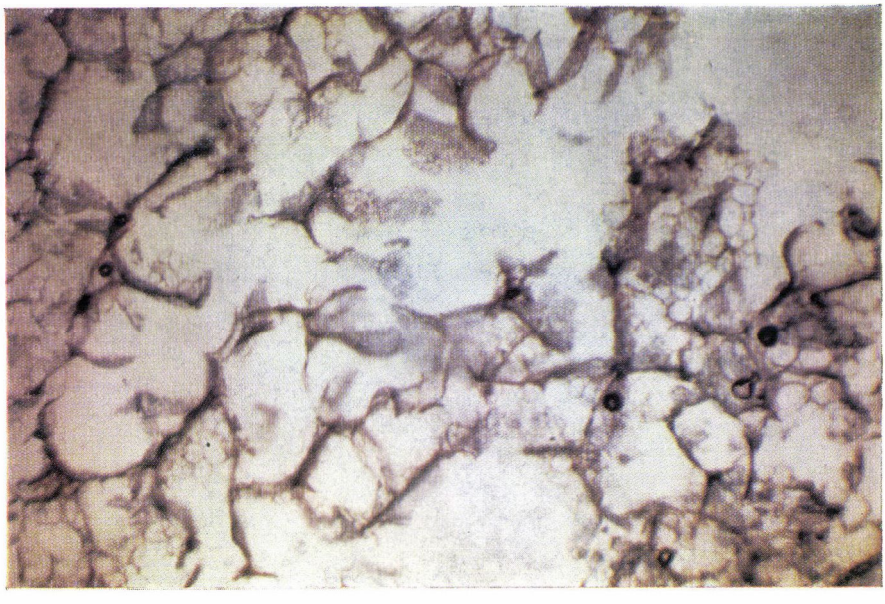

Fig. 11. Peroxidase localization in potatoes of the variety "Gül baba". Experimental conditions as in Fig. 10

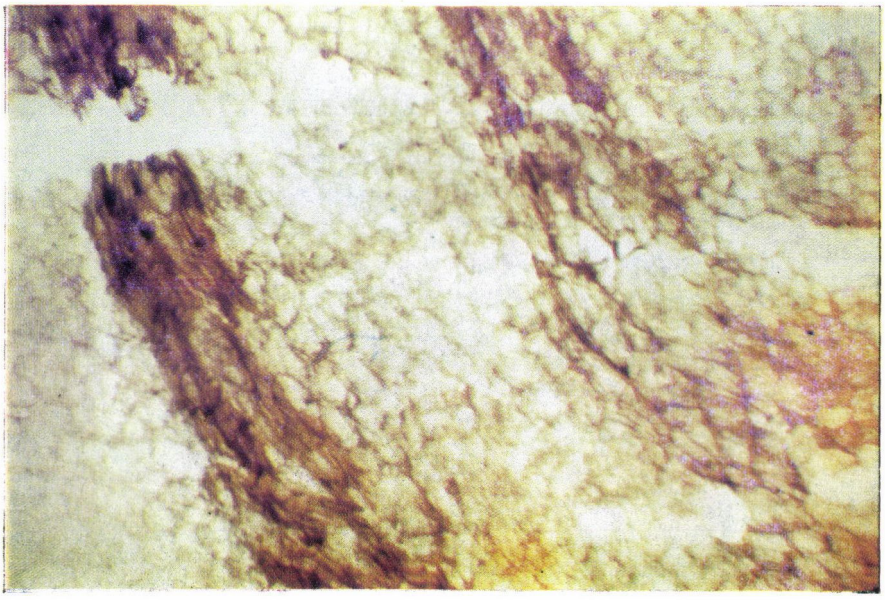

Fig. 12. Peroxidase localization in head of celery. Experimental conditions as in Fig. 10

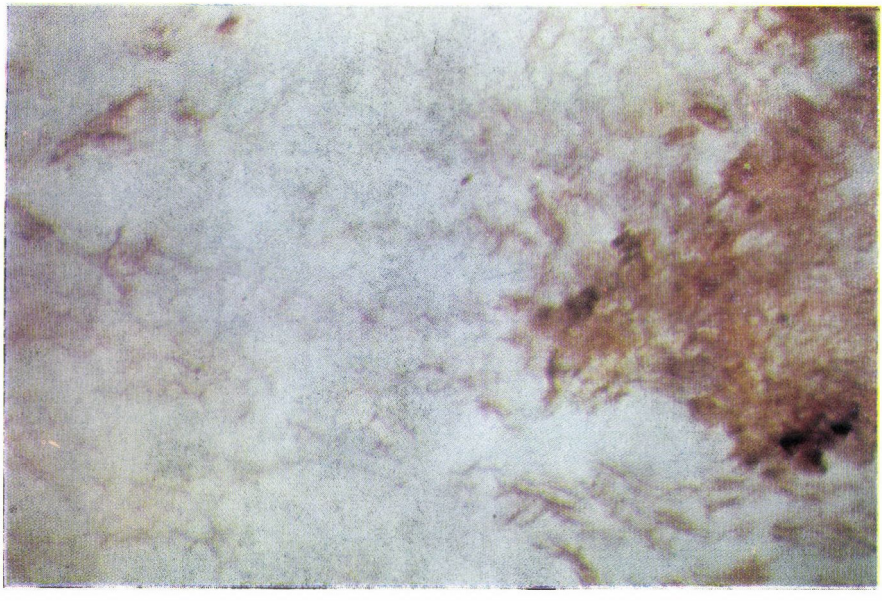

Fig. 13. Peroxidase localization in head of celery. Experimental conditions as in Fig. 10

3. Conclusions

The method of measurement developed lends itself to the determination of peroxidase activity of vegetables on a strictly kinetic basis, since the changes in *OD* as induced by enzyme action are in a linear relationship to reaction time (Fig. 3). Enzyme activity may be characterized by initial reaction rate, *i.e.* the change in *OD* per min. Unit activity can be expressed in terms of change in *OD* as arbitrarily adopted. Peroxidase activity of vegetables may be expressed related to 1 g of product, *i.e.* in terms of enzyme concentration as well. The method permits further to compare changes in *OD* per min with a reference enzyme of known activity and thus to express enzyme activity in international units (WINTER, 1968).

The method of activity measurement is of high accuracy, coefficients of variation of repeated determinations from different potato homogenates were below 5%.

According to the investigations described here reaction rate of potato peroxidase has a maximum at a H_2O_2 concentration of 0.025% (w/v) ($7.35 \cdot 10^{-3}$ *M*) (Fig. 1). The inhibition of peroxidase by its substrate has been observed with a preparation from horseradish by WEINRYB (1966), too, however, at much higher hydrogen peroxide concentrations (>1 *M*). This seems to indicate that inhibition depends on the ratio of enzyme and substrate in the reaction mixture. Differences in characteristics originating from the enzyme source may also account for the different thresholds of substrate inhibition. It is interesting to note that with potato peroxidase even the rising section of the curve, obtained on plotting reaction rate *vs.* substrate concentration, was found to be divergent from the ideal (hyperbolic) shape.

Thus, while optimum substrate concentration as established for potato peroxidase (0.025%, w/v) seemed appropriate to measure the activities of several kinds of vegetables, it may be expedient to determine the respective values individually for every product to ensure substrate saturation on the one hand and to avoid substrate inhibition on the other.

No relationship was found between the natural pH of the product (Table 1) and its enzyme concentration as established at pH 5.0. *E.g.* the difference in the enzyme concentration values of turnips and winter kohlrabi, both of identical pH (6.12) was found to be more than 50-fold.

Peroxidase activity of a given product is, on the contrary, highly dependent on variations in pH as was shown for the potato enzyme (Fig. 5). pH values around 5 seem to be the optimum for other products as well. The activity of soybean peroxidase during ripening of the plant was found to be more than twice as high in reaction mixtures of pH 5.0 than in those of pH 6.5 (RACKIS *et al.*, 1972). A peroxidase preparation isolated from a palm-tree species *(Anona squamosa)* by Indian authors (SASTRY *et al.*, 1961) showed the

highest activity in the pH-range of 5.0 to 5.5. The inactivation of horseradish peroxidase in acidic medium seems to be dependent not only on pH but also on the compound used for acidification (EMBS & MARKAKIS, 1969): an exposure of the enzyme to pH 4.0 for 30 h caused greater losses in activity when sulfuric acid was applied than could be observed under identical conditions with sodium hydrogen sulfite.

Heat resistance of peroxidase is the most studied characteristic of the enzyme, owing to its great importance in the canning and allied industries. In the blanching process temperature and duration of exposure should be selected so as to preserve nutritional value and prevent the formation of off-flavours beside of inactivating undesirable oxidative enzymes (VOIROL, 1972). This implies the knowledge of heat resistance values for the peroxidases of every product. The experiments described here show considerable differences in this respect between the various products. While potato peroxidase is completely inactivated by a 10-min heat treatment at 95 °C, the kohlrabi enzyme retains some of its activity even after exposures, for the same period of heating, to 100 or 110 °C and is reactivated to a considerable extent on subsequent keeping at room temperature. Similar differences in heat resistance were established by WINTER (1968) for a number of other products (mushrooms, asparagus, cauliflower, spinach and green peas).

The behaviour of some products suggests studying the relationship between lignin formation and peroxidase activity. Investigations to that effect have been carried out by RANADIVE and HAARD (1972) with different varieties of pears. These authors demonstrated by means of histochemical tests that highest concentrations of peroxidase occurred in those parts of the pear fruit in which the lignin-containing "stone cells" were localized, *i.e.* in the pericarp, in the parts near the skin, in the parenchyme cells surrounding the granules and in the fruit flesh along the cell walls. The existence of a similar relationship in vegetables of higher lignin content (kohlrabi, radish) is indicated by the fact that the enzyme concentration of the early product is much lower. *E.g.* peroxidase concentration in early kohlrabi is about 1/4 as compared to the stringy winter product, while in early radish it is 200 times lower than in black radish (Table 1).

In apricots, grapes and prunes the parts around the skin and stone, resp., were reported to have the highest peroxidase content (VAS, 1954). The results presented here show a similar localization of the enzyme in vegetables such as black radish and head of celery (Figs. 10, 12 and 13).

The data so far obtained shall serve as starting point in the development of efficient inhibition processes of the enzyme.

*

The authors are indebted to Head Physician Dr. D. TANKA and Chemical Engineer Dr. M. KELLER of the Histochemical Laboratory of the NATIONAL INSTITUTE OF RHEUMATISM AND PHYSIOTHERAPY for their assistance in the histochemical investigations. Thanks are due to GY. BENDÓ for skilful laboratory work.

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Acta Alimentaria, Vol. 4 (3), pp. 309—329 (1975)

PRODUCTION OF CHEESE WITH A MILK CLOTTING ENZYME PREPARATION OF MICROBIAL ORIGIN

PART I. FATTY ACID COMPOSITION OF CHEESE

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(Received September 11, 1974)

Investigations into the free and total fatty acid content as well as into the quantitative distribution of the acids in the range of 2 to 10 carbon atoms were carried out by steam distillation and gas chromatography with the ultimate aim to compare cheeses manufactured in parallel with calf rennet and a microbial enzyme preparation developed in this laboratory. The presence of C_{11}—C_{16} fatty acids could also be detected by the method.

The various cheese types investigated, as manufactured with calf rennet, were considerably different from each other with respect both to free fatty acid content and to the ratio of free to total acidity.

9 to 14 fatty acids of even and uneven numbers of carbon atoms could be detected in the various cheese types, the latter being present in most cases in small amounts only. Among the fatty acids identified no unsaturated or branched-chain compound was found. It was established that the various cheese types could be characterized by the presence of certain fatty acids in exceedingly high amounts and by the absence of others, resp.

With the exception of White cheese the batches manufactured with microbial rennet differed in free fatty acid content and/or distribution of C_2—C_{10} fatty acids from those produced in parallel with calf rennet. No difference was found in the number of chromatographically detectable fatty acids when applying optimum concentration of microbial rennet in cheese making.

The differences in fatty acid content and composition as obtained by chemical analysis were not reflected in the sensory characteristics of the products.

A process has been developed in this laboratory to produce a milk clotting enzyme by submerged fermentation of an *Endothia parasitica* strain. The enzyme has been obtained both as liquid and as powdered preparation. The preparations were tested in pilot plant and industrial scale cheese making experiments for their suitability to manufacture hard, semi-hard and soft cheese from cow's milk and the soft-type White cheese from ewe's milk. The cheese samples produced in these trials were thoroughly investigated by chemical and sensory methods and results were compared to those obtained for the same types of cheese manufactured in parallel with calf rennet. An account on this comparative study will be given in a series of papers dealing with investigations into fatty acid composition, total and soluble protein as well as free amino acid content of the different cheese types and, finally, giving a description of the cheese making processes as applied with the different kinds of rennets along with the results of sensory evaluation.

The first paper deals with gas chromatographic estimation of fatty

acids, since these are — whether volatile or non-volatile, free or present as salts — important components of cheese flavour (LANGLER & DAY, 1966). As reported by several authors, different cheese types have distinct and characteristic fatty acid patterns (SCHORMÜLLER & LANGNER, 1960; NEY & WIROTAMA, 1971, 1972; ADDA & DUMONT, 1974). These may vary, however, according to season, raw material (milk) and production technology (SCHORMÜLLER & LANGNER, 1960; LANGLER & DAY, 1966). No data of this kind have been published so far on Hungarian cheese types. In a previous paper (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974) a combined method of steam distillation and gas chromatography has been described for estimating the C_2—C_8 straight chain saturated fatty acids of Swiss-type (Emmental) cheese. The applicability of the method has since been extended to separate fatty acids up to 14 or 16 carbon atoms present as free acids or salts in hard, semi-hard and soft cheese. This implied some modifications of the procedure originally developed which will be given below along with the fatty acid patterns of 5 cheese types marketed in Hungary as influenced by the type of rennet applied in the manufacturing process.

1. Materials and methods

1.1. Cheese types

Four types of cheese manufactured from cow's milk as well as the soft-type White cheese manufactured from ewe's milk were investigated. The four cheese types obtained from cow's milk included a hard (Emmental), a semi-hard (Trappist) and two soft types ("Tea"-cheese and "Pálpusztai"). The periods of ripening (in days) of these types are as follows: 90 (Emmental), 30 (Trappist), 21 ("Tea"-cheese), 14 ("Pálpusztai") and 6 (White cheese).

One to five batches each of these cheese types manufactured with calf stomach rennet (*CR*) and liquid or powdered microbial rennet (*MR*) at different factories according to standard technology were analyzed at the mature state, *i.e.* after the periods of ripening indicated above, irrespective of the type of rennet used. The effect of the clotting agent on fatty acid composition was studied with cheese batches manufactured simultaneously from the same raw material under identical conditions with calf rennet and with the microbial preparation, resp. Identical treatment of the batches produced in parallel was maintained during the ripening process as well. Emmental was manufactured at the ZALAEGERSZEG CHEESE FACTORY, the semi-hard and soft cheeses from cow's milk at the training shop of the CSERMAJOR DAIRY ENGINEERING SCHOOL and White cheese at the MEZŐKÖVESD CHEESE PLANT.

In some cases samples from retail shops were investigated to establish the average fatty acid composition of the different cheese types as obtained when using calf rennet.

1.2. Extraction and determination of free and total acidity

Free fatty acids (*FFA*) as well as those present as salts were extracted by steam distillation. Contrary to the procedure described earlier (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974) for estimating the C_2—C_8 free fatty acid content of cheese, steam distillation was carried on until total recovery of the acids in the distillate. (When estimating only C_2—C_8 acids, recovery of about 80% of the total acid content was sufficient to yield a gas chromatographic distribution pattern not differing significantly from the one obtained on 100% recovery. The reason for this phenomenon can be easily understood from Figs. 1 and 2.)

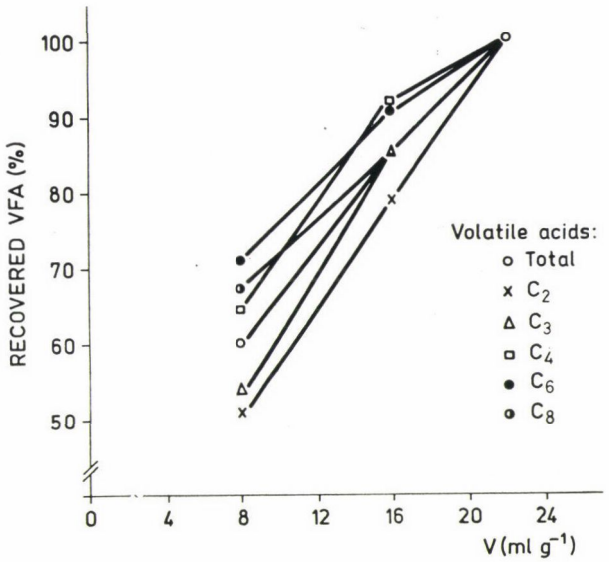

Fig. 1. Relationship between the volume of distillate (V) related to cheese weight and recovery of volatile fatty acids (VFA) on steam distillation. Total free volatile acidity: 4.13 meq/100 g cheese (commercial sample of Emmental)

The amount of distillate to be collected for total recovery depends on the fatty acid content of cheese and had, therefore, to be determined in preliminary experiments for every type. Table 1 contains the volumes of distillate and the sample sizes of grated cheese as applied at various levels of total and total free acidity, resp. In order to extract total acidity, 50% phosphoric acid was added in a ratio of 1 : 1 (w/v) to the cheese sample prior to steam distillation.

A glass assembly as designed by PETRÓ-TURZA & SPANYÁR (1969) was applied to accelerate steam distillation (Fig. 3).

The acids were collected in a known excess of 0.1 *N* NaOH (20—50 ml) and 50 ml aliquots of this solution were used to determine total (or total free)

acidity by titration with 0.01 *N* HCl in the presence of phenolphthalein. Acidity was expressed in meq and related to 100 g of cheese.

The rest of the distillate was used to prepare the sample for gas chromatography.

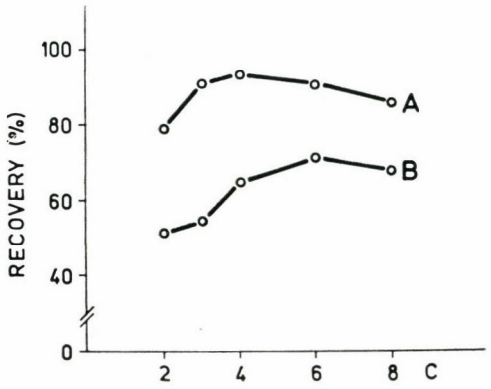

Fig. 2. Relationship between the number of carbon atoms (C) and the recovery of individual volatile fatty acids on steam distillation at various levels of recovery of total acidity. Total free volatile acidity: 4.13 meq/100 g cheese (commercial sample of Emmental). *A* — volume of distillate 16 ml g^{-1} cheese, recovery of total free volatile acidity 85%; *B* — volume of distillate 8 ml g^{-1} cheese, recovery of total free volatile acidity 60%

Table 1

Sample sizes of cheeses of various total acidities and volumes of steam distillate to be collected for total recovery of fatty acids

Total (or total free) acidity, meq	Sample size (g)	Volume of distillate	
		ml	related to sample size (ml g^{-1})
< 4	25.0	750	30
4 — 6	12.5	500	40
6 — 8	12.5	750	60
8 — 10	10.0	1 000	100
>10	5.0	1 000	200

1.3. Preparation of the sample for gas chromatography

The preparation of the sample was carried out exactly as described earlier (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974). 1 μl portions of the ether solution of the liberated fatty acids were injected into the gas chromatograph.

1.4. Gas chromatography

Separation of the fatty acids was performed in a PERKIN-ELMER 900 gas chromatograph equipped with a dual flame ionization detector. Columns of 3.6 m length and 2 mm inner ∅ were used with 80—100 mesh silanized Chromosorb W carrier, coated with 10% polyethylene glycol as stationary phase.

Temperature programming at a rate of 5 °C min^{-1} was applied in the range of 130—180 °C. The temperature of the injection port was 200 °C, that of the detector 240 °C. Carrier gas flow (N_2) was adjusted to 35—60 ml min^{-1}. Attenuation was varied between 8- and 16-fold, paper displacement rate was 5 mm min^{-1}.

The acids were identified by their retention distances, partly using known standards and partly on the basis of the linear relationship existing under isothermal conditions (180 °C) between the logarithm of the retention distance and the number of the carbon atoms.

Volatile acid distribution was calculated from peak areas and expressed as percentage of total area. On the average 3 samples were taken from each cheese and 2 parallel gas chromatograms run from each sample. Mean values of parallel determinations and standard deviations were calculated. Results were compared by *Student*'s t test.

2. Results

2.1. Total and total free acidity of different cheese types

Total free acidity of all the cheese types investigated as manufactured with calf rennet and total acidity of Emmental and "Tea"-cheese are summarized in Table 2.

Table 2

Total and total free acidity of different cheese types marketed in Hungary

Name of cheese	pH+	Total free acidity, meq/100 g cheese		Total acidity, meq/100 g cheese		Acids present as salt, meq/100 g cheese
		$\bar{x}$	s	$\bar{x}$	s	
Emmental	—	2.25	0.54	6.90	0.93	4.65
Trappist	5.65	5.96	0.24	—	—	—
"Tea"-cheese	5.35	3.49	0.50	5.47	0.24	1.98
"Pálpusztai"	6.00	1.94	0.00	—	—	—
White cheese	4.55	7.62	0.44	—	—	—

+ pH was determined with a *Metrohm* E 166 type pH-meter
$\bar{x}$ = mean
s = standard deviation
Number of parallel steam distillations = 3—5

As can be seen cheese types differ considerably in total free acidity as obtained on steam distillation: the highest value, 7.62 meq/100 g can be found in White cheese, the lowest, 1.94 meq/100 g in the soft cheese "Pálpusztai" prepared from cow's milk. The ratio of the two values is about 4 : 1. An inverse parallelism may be noted between pH and free acidity of White cheese and

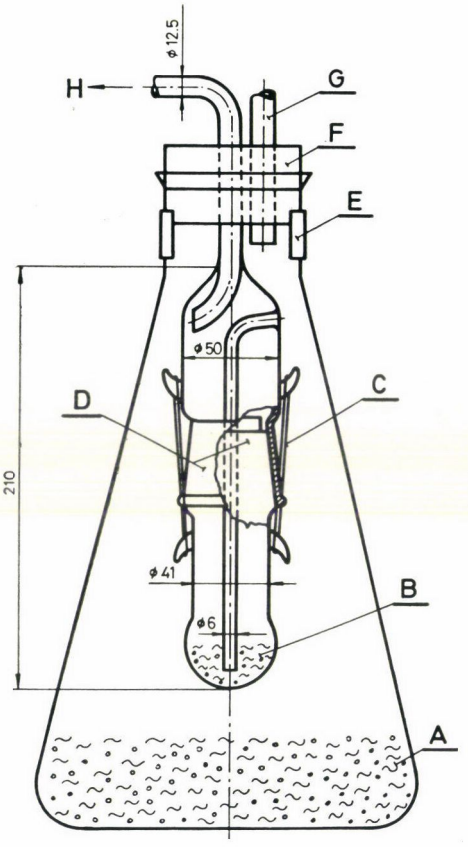

Fig. 3. Steam distillation assembly. *A* — steam generator, *B* — sample, *C* — elastic fasteners, *D* — ground glass connection, *E* — metal clamps, *F* — rubber stopper, *G* — pressure equalizer, *H* — connection to receiver

cheeses from cow's milk: White cheese of the lowest pH had the highest and "Pálpusztai" of the highest pH the lowest free acid content, while "Tea"-cheese proved medium in both respects. Trappist cheese is an exception to the rule. However, it has to be borne in mind that this cheese was ripened in plastic foil and cannot be compared to the other types manufactured by the traditional procedure. The pH value of the Emmental sample enlisted in the table has not been determined. According to measurements carried out in this

laboratory with other samples of this cheese type (KISS *et al.*, 1973) as well as to data from the literature (LANGLER & DAY, 1966) its value is between 5.4 and 5.8 which fits in well with the free acid content of 2.25 meq.

Total acidity has been determined in Emmental and "Tea"-cheese. In both types its values were found to be considerably higher than those of free acidity. The difference of these values, *i.e.* the amount of acids in salt form is nearly the double of free acidity in Emmental, whereas in "Tea"-cheese it amounts but to about 70% of the latter. Thus in Emmental the greater part of the total acid content is present as salts, while in "Tea"-cheese as free acids.

2.2. Free fatty acid composition

The gas chromatographic free fatty acid patterns of the different cheese types as manufactured with calf rennet are represented in Figs. 4—8.

The chromatograms show qualitative as well as quantitative differences between the different cheese types. For the sake of comparison the free fatty acids found in the steam distillates of the individual cheese types are summarized in Table 3.

Table 3

Free fatty acids of different cheese types as detected by gas chromatography

Acid \ Cheese type	Emmental	Trappist	"Tea-cheese"	"Pálpusztai"	White cheese
Acetic	+	+	+	(+)	+
Propionic	+	(+)	+	+	(+)
Butyric	+	+	+	+	+
Unknown I	–	–	–	–	(+)
Hexanoic	+	+	+	+	+
Unknown II	–	–	–	–	+
Octanoic	+	+	+	+	+
Nonanoic	–	–	–	(+)	+
Decanoic	+	+	+	+	+
Undecanoic	(+)	(+)	(+)	–	+
Dodecanoid	+	+	+	+	+
Tridecanoic	(+)	–	(+)	–	(+)
Tetradecanoic	+	+	+	+	+
Hexadecanoic	+	+	–	–	+
Total number of acids	11	10	10	9	14

\+ acid present in measurable amounts
(+) acid present in traces
– acid not detectable

As can be seen, the majority of the acids present in measurable amounts in the free fatty acid extracts were straight chain saturated compounds with even numbers of carbon atoms. Of the fatty acids with uneven numbers of carbon atoms propionic acid was present in all the cheese types, although only Emmental and "Pálpusztai" contained it in measurable amounts. An un-

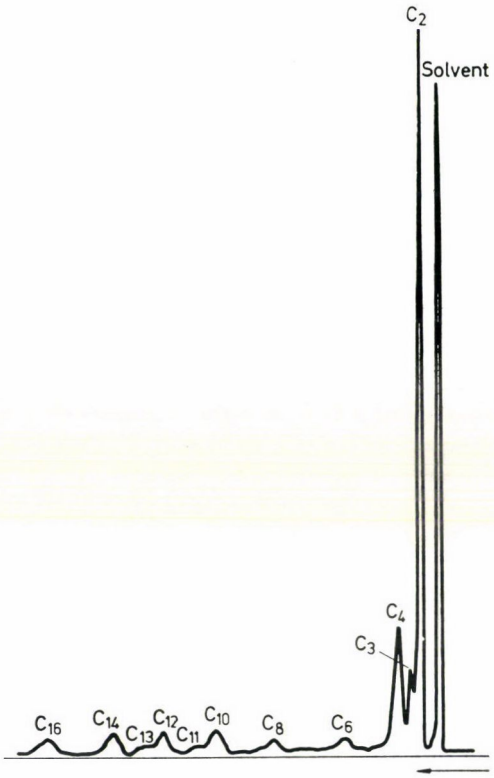

Fig. 4. Gas chromatogram of the free fatty acids (*FFA*) of Emmental cheese manufactured with calf rennet. Experimental conditions: extract of *FFA* prepared by steam distillation according to Table 2, subsequent evaporation to dryness as Na salts, followed by liberration of acids with H_2SO_4 and dissolving in diethyl ether, elimination of traces of water by anhydrous Na_2SO_4 and evaporation of solvent. 1 μl of *FFA* extract injected into the gas chromatograph. Gas chromatography: PERKIN-ELMER 900, dual flame ionization detector; column length 3.6 m, inner ⌀ 2 mm; support: 10—12 g 80—100 mesh Chromosorb W treated with dimethyl chlorosilane; stationary phase: 10% polyethylene glycol adipate; temperature programme: 5 °C min^{-1} from 130 to 180 °C, injection port 200 °C, detector 240 °C; N_2 carrier gas flow 35—60 ml min^{-1}, attenuation 8- to 16-fold, paper displacement rate 5 mm min^{-1}. Acids: C_2 = acetic, C_3 = propionic, C_4 = butyric, C_6 = hexanoic, C_8 = octanoic, C_{10} = decanoic, C_{11} = undecanoic, C_{12} = dodecanoic, C_{13} = tridecanoic, C_{14} = tetradecanoic, C_{16} = hexadecanoic

identified compound, probably heptanoic acid (marked ++ in Fig. 8) as well as nonanoic acid were present in fair proportions in White cheese, traces of the latter were detected in "Pálpusztai", too. Undecanoic acid could be found in

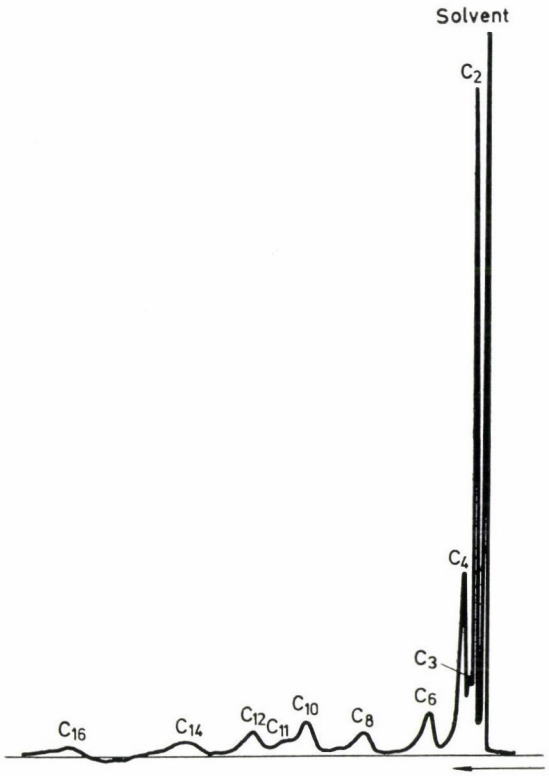

Fig. 5. Gas chromatogram of the free fatty acids of Trappist cheese manufactured with calf rennet. Experimental conditions: see Fig. 4

all the types but "Pálpusztai", although — with the exception of White cheese — only in traces. Tridecanoic acid was detected in trace amounts only and was entirely lacking from the chromatograms of "Pálpusztai" and Trappist.

Table 4

Characteristic free fatty acid patterns of different cheese types

Name of cheese	Characteristic fatty acids			
	Acetic	Propionic	Butyric	Decanoic
Emmental	×		+	
Trappist	×	–	+	+
"Tea"-cheese	×		+	×
"Pálpusztai"	–	+	×	
White cheese	×	–		– +

× acid present in the highest amount
\+ acid present in considerable amount
– acid present in traces or not at all
No sign is applied for acids not considered characteristic of the respective cheese type.

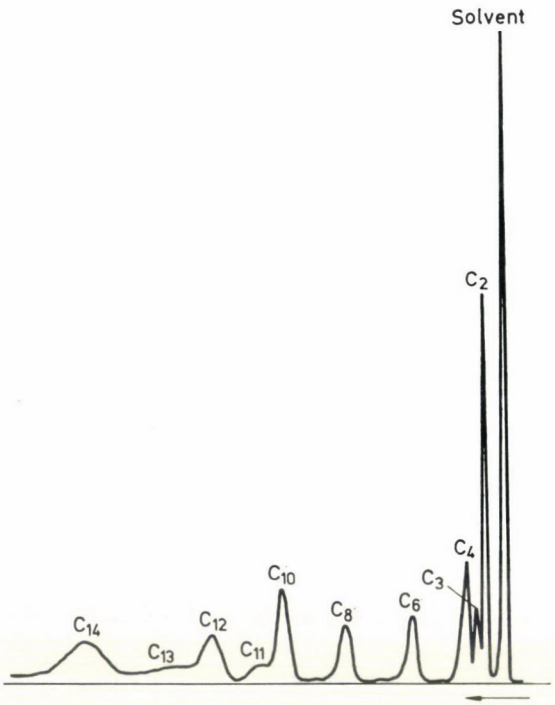

Fig. 6. Gas chromatogram of the free fatty acids of "Tea"-cheese manufactured with calf rennet. Experimental conditions: see Fig. 4

Considering the main acids present in the highest amounts or lacking from the respective chromatograms, characteristic fatty acid patterns may be established for the five cheese types investigated. These are given in Table 4.

2.3. Distribution of total and total free acidity

A hard and a soft type cheese (Emmental and "Tea"-cheese), resp., both manufactured from cow's milk and with calf rennet, were selected to gain information on the composition of total fatty acids as compared to the free ones. Fig. 9 shows the distribution of the C_2—C_{10} total and free fatty acids present in measurable amounts (*i.e.* yielding distinct chromatographic peaks) in the steam distillates of the above cheese types.

As can be seen, significant differences in free and total fatty acid distribution were found only with respect to two acids in each cheese type (propionic and butyric or hexanoic). The characteristic fatty acid patterns of the cheeses as established in Table 4 were not altered by these differences. Thus it may be concluded that investigations into free and total fatty acid distributions, resp., equally lend themselves to the objective of this study, *i.e.* to the

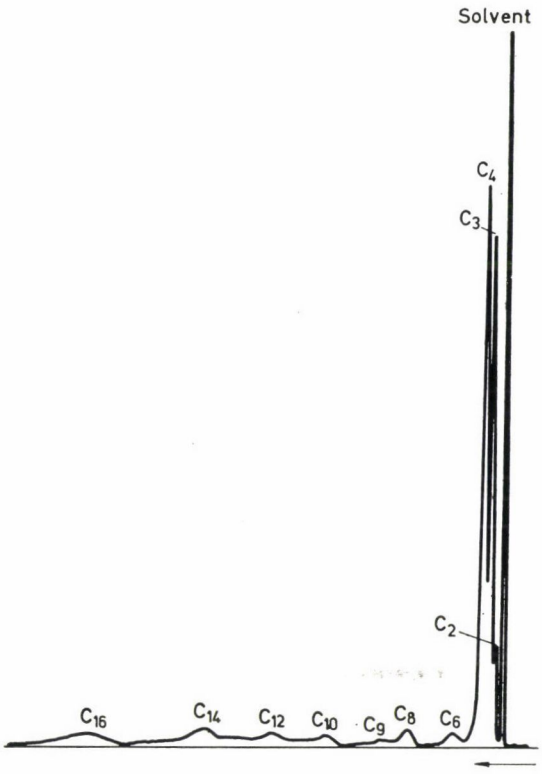

Fig. 7. Gas chromatogram of the free fatty acids of "Pálpusztai" cheese manufactured with calf rennet. Experimental conditions: see Fig. 4; C_9 = nonanoic acid

comparison of cheeses manufactured with different types of rennet. For technical reasons the former was adopted for subsequent work.

2.4. Effect of the renneting agent on the free fatty acid composition

The values of free acidity as established for cheese manufactured in parallel with *CR* and *MR*, resp., are shown in Fig. 10.

The control cheese samples manufactured with *CR* as illustrated in Fig. 10 are only partly identical with those of Table 1, the respective acid contents are, nevertheless, similar. The data concerning the cheeses manufactured with *MR* are mean values obtained from two batches (with the exception of "Pálpusztai" cheese, of which only one batch was analyzed).

The data point to different effects of the renneting enzymes on total free acidity of the various cheese-types. Free acidity of Emmental manufactured with *MR* was a multiple of the value found in the control batches, while, on the contrary, with Trappist cheese the acid content was higher in the latter

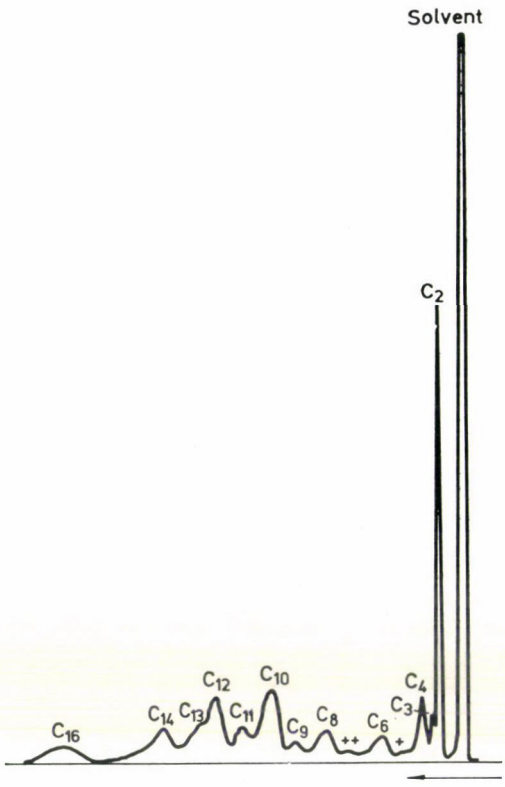

Fig. 8. Gas chromatogram of the free fatty acids of White cheese manufactured with calf rennet. Experimental conditions: see Fig. 4; C_9 = nonanoic acid, + and ++ = acids not identified

(about double). In "Tea"-cheese, too, the free fatty acid content was higher in the control batch, whereas in "Pálpusztai" and White cheese no significant difference in acidity was noted with different renneting agents.

The chromatographic fatty acid patterns of the cheese batches manufactured with the optimum concentration of *MR* as established by KISS and co-workers (1974) were essentially identical with the ones obtained for the control samples (Figs. 4—8 and Table 3). A comparison of quantitative distributions of the acids present in measurable amounts is given in Figs. 11—15.

In the case of Emmental cheese, as was shown with two batches each in a previous paper (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974), utilization of *MR* brought about a significant change in the quantitative distribution of volatile free fatty acids. This could now be confirmed with a larger number of batches (4, partly commercial batches manufactured with *CR* and 5, exclusively experimental ones produced with *MR*). Fig. 11 shows the relative amount of propionic acid to be on the average 3 times higher in cheeses manufactured with

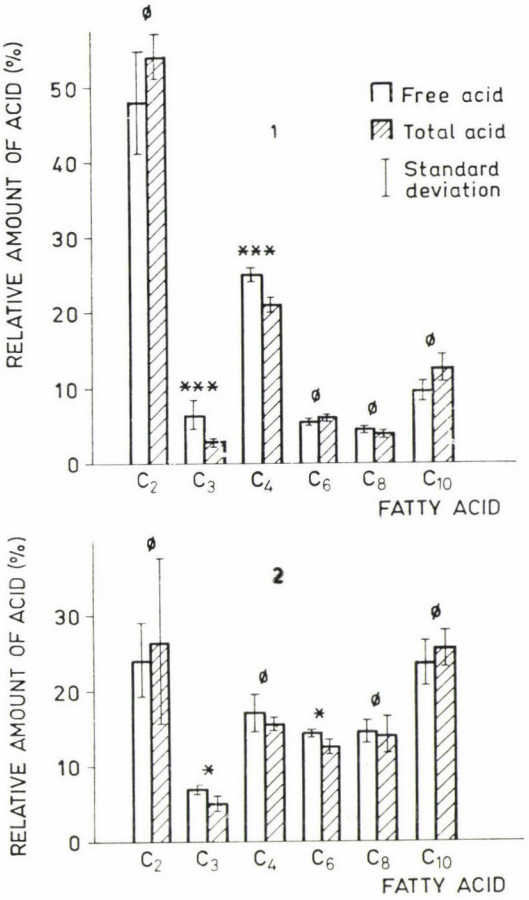

Fig. 9. Distribution of free and total C_2—C_{10} fatty acids, resp., of Emmental (1) and "Tea"-cheese (2). Experimental conditions of analysis as in Fig. 4. In steam distillation of total fatty acids water added to the cheese samples was replaced by 50% phosphoric acid. * and *** indicate differences significant at the probability levels of 95 and 99.9%, resp. Ø = difference not significant

MR than in the control batches, while the respective contents of acetic, butyric and caproic acids are correspondingly lower in the former. The differences are significant in spite of the high standard deviations.

FFA distributions of Trappist batches (Fig. 12) manufactured with *CR* and *MR*, resp., differ significantly only with respect to their relative decanoic acid contents: in cheese manufactured with *MR* this compound does not belong to the main fatty acid components as given in Table 4. The decrease in the relative decanoic acid content and the corresponding increase in the respective value of butyric acid in cheese manufactured with *MR* were equally observed in batches ripened in plastic foil and in those ripened according to traditional technology (Kiss *et al.*, 1973).

In the case of "Tea"-cheese (Fig. 13) striking differences were found between the batches obtained with the two different rennets. The relative butyric acid content was more than twice as high in the batches manufactured with *MR* than in the control sample, while the relative C_2—C_{10} fatty acid contents were accordingly lower in the experimental ones. In the control batch (Fig. 6) the overall content of acetic and decanoic acids, present in a

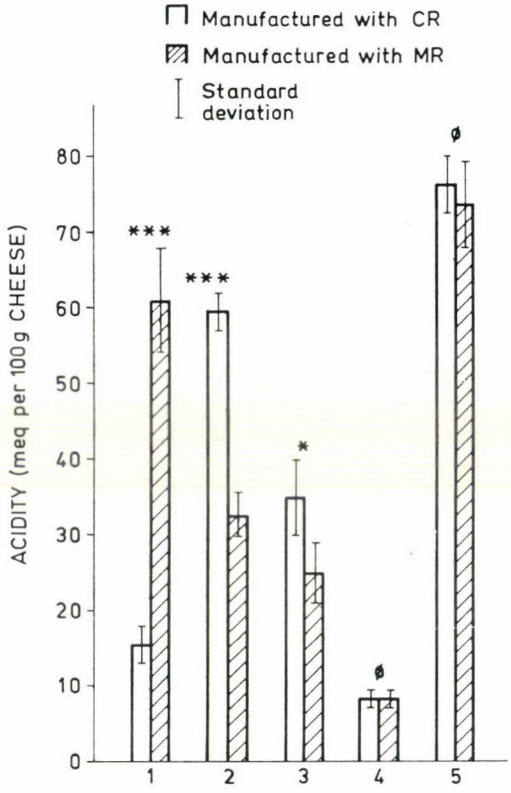

Fig. 10. Effect of the type of rennet on the free fatty acid content of different cheese types. *1* — Emmental, *2* — Trappist, *3* — "Tea"-cheese, *4* — "Pálpusztai", *5* — White cheese. Experimental conditions of analysis as in Tables 2 and 3, resp. * and *** indicate differences significant at the probability levels of 95 and 99.9%, resp. Ø = difference not significant. *CR* = calf rennet, *MR* = microbial rennet

ratio of about (1 : 1) amounted to 47% of the C_2—C_{10} fatty acid content, while butyric acid constituted only 17%. In the batches produced with *MR* the relative butyric acid concentration was higher than 40%, while another 40% were made up by acetic and decanoic acids, present in a ratio of 3 : 1.

Considering the *FFA* composition of "Pálpusztai" cheese, comparison was restricted to the respective propionic and butyric acid contents, since the

other compounds separated by gas chromatography were present in negligible amounts only. It is clear from Fig. 14 that the ratio of these two acids varies considerably with the type of rennet applied: in the batch manufactured with *MR* the relative propionic acid content is higher.

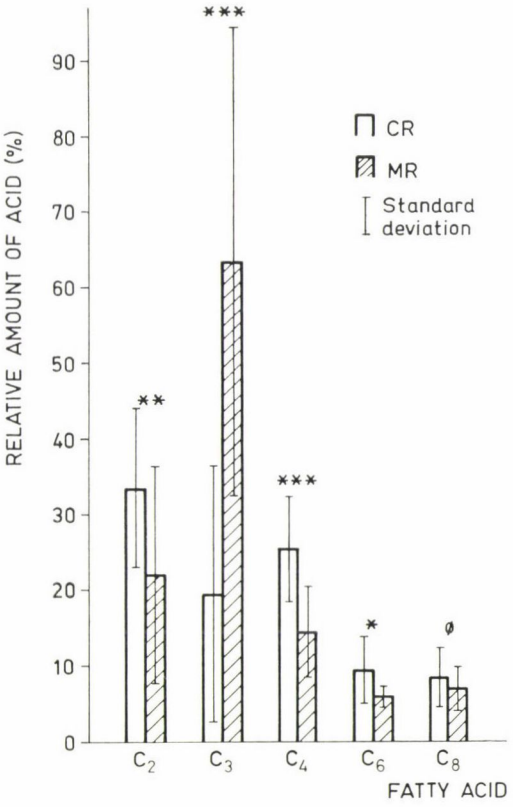

Fig. 11. Distribution of the C_2—C_8 *FFA* of Emmental cheese manufactured with calf rennet (*CR*) and microbial rennet (*MR*), resp. Experimental conditions of analysis as in Fig. 4. *, ** and *** indicate differences significant at the probability levels of 95, 99 and 99.9%, resp. Ø = difference not significant

The comparison of the C_2—C_{10} *FFA* of White cheese did not yield any significant difference between the batches manufactured with *CR* and *MR*, respectively.

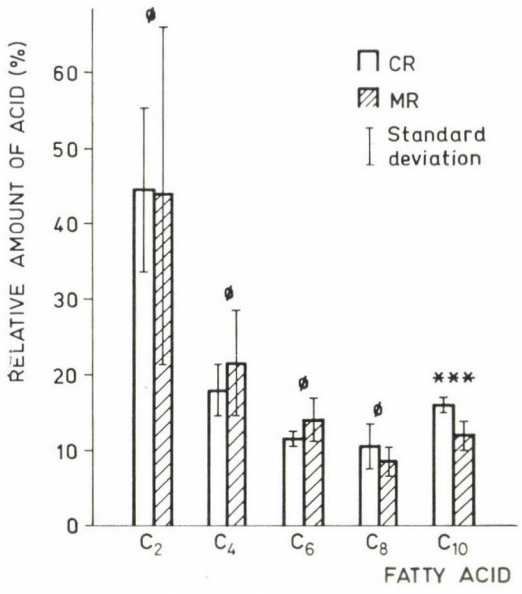

Fig. 12. Distribution of the C_2—C_{10} *FFA* of Trappist cheese manufactured with calf rennet (*CR*) and microbial rennet (*MR*), resp. Experimental conditions of analysis as in Fig. 4. *** = difference significant at the probability level of 99.9%. Ø = difference not significant

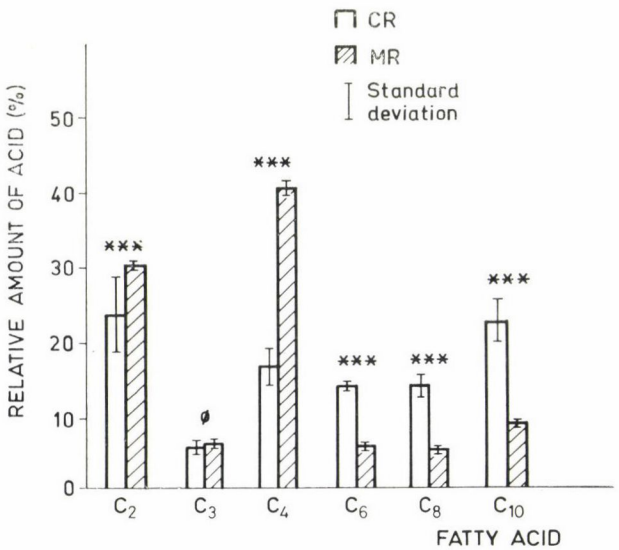

Fig. 13. Distribution of the C_2—C_{10} *FFA* of "Tea"-cheese manufactured with calf rennet (*CR*) and microbial rennet (*MR*), resp. Experimental conditions of analysis as in Fig. 4. *** = difference significant at the probability level of 99.9%. Ø = difference not significant

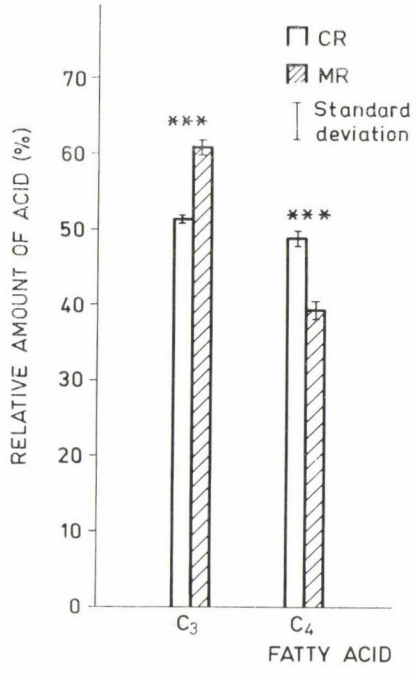

Fig. 14. Distribution of C_3—C_4 *FFA* of "Pálpusztai" cheese manufactured with calf rennet (*CR*) and microbial rennet (*MR*), resp. Experimental conditions of analysis as in Fig. 4. *** indicates differences significant at the probability level of 99.9%

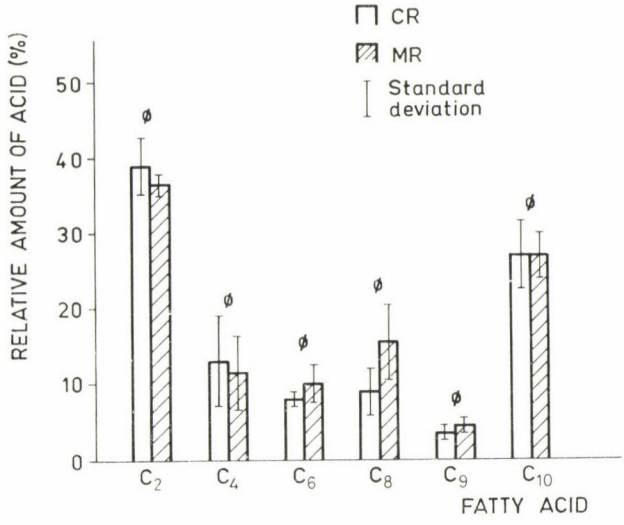

Fig. 15. Distribution of C_2—C_{10} *FFA* of White cheese manufactured with calf rennet (*CR*) and microbial rennet (*MR*), resp. Experimental conditions of analysis as in Fig. 4. Ø = difference not significant

3. Conclusions

Data on the fatty acid composition of the cheese types dealt with in this paper are practically not available in the literature with the exception of those concerning Emmental cheese (JAGER, 1967; KIERMEIER *et al.*, 1968a, b; LANGLER & DAY, 1966; MAYR, 1969; NEY & WIROTAMA, 1972; PATTON, 1964; PANOUSE *et al.*, 1972). In this Institute the distribution of C_{10}—C_{16} *FFA* with even numbers of carbon atoms was investigated in Emmental cheese by KEVEI-PICHLER and co-workers (1969) applying gas chromatography of the corresponding methyl esters. The data presented by these authors on the relative amounts of the above acids are in good agreement with the findings of others (LANGLER & DAY, 1966). Thus it seemed more promising to focus the present investigations on the low molecular weight volatile acids. Data as to the effect of milk clotting enzymes on the volatile acid composition of cheese were published by SVYAGINTSEV and co-workers (1972). These are concerned, however, with an enzyme preparation obtained from a *Mucor*-strain and applied to manufacture but one type of cheese ("Kostromskii"). The results reported showed an increase in both acetic and butyric acid contents of the experimental lots as compared to control samples manufactured with calf rennet.

Quantitation of gas chromatographic data was carried out in the present study with acids up to C_{10}, although some authors extend the term of volatility up to C_{14} (DAVIS, 1966). Comparison of the acid distributions found for Emmental with the findings of others (KEVEI-PICHLER *et al.*, 1969; LANGLER & DAY, 1966) made it, however, obvious that extraction by steam distillation of acids with more than 10 carbon atoms cannot be considered quantitative. Taking into account the comparative character of this study qualitative information with respect to differences in higher fatty acid composition (up to C_{16}) was thought to be valuable, too.

Several authors report on the relationship betwen pH and the ratio of free and total fatty acids and on the total fatty acid content of Emmental cheese. Considering the pH of this cheese type, 60 to 90% of its fatty acid content may be expected to be present as salt, as pointed out by LANGLER and DAY (1966). The results described here are in agreement with the above findings, in that 67% of the total acidity of Emmental was found to be present in the salt form (Table 2). Values published on total C_2—C_6 and C_2—C_8 fatty acid content in 100 g of Emmental amounted to 8.6 and 14.8 meq, resp. (KIERMEIER *et al.*, 1968a; LANGLER & DAY, 1966), and are considerably higher than those established in this laboratory (6.9 meq).

The characteristic differences in *FFA* composition of different cheese types as established by gas chromatography (Figs. 4—8, Tables 3 and 4) may be of interest from the scientific point of view. Especially the considerable

divergence of the *FFA* composition of the Emmental batches manufactured with *CR* from the generally accepted pattern seem to merit more close attention. Propionic fermentation is considered characteristic of Emmental of good quality and propionic acid is reported to be the main fatty acid component of this cheese (KIERMEIER *et al.*, 1968a). The ratio of acetic and propionic acids was established by several authors (JAGER, 1967; KIERMEIER *et al.*, 1968a; LANGLER & DAY, 1966; PATTON, 1964) to vary between 1 : 0.6 and 1 : 12, while butyric acid was usually found only in minute amounts (in extreme cases, however, it constituted 28—29% of the propionic acid content). In the Emmental samples manufactured with calf rennet the present investigations established the ratio of $C_2 : C_3 : C_4$ acids as 1 : 0.6 : 0.8, *i.e.* the propionic acid content as related to acetic acid was found to correspond to the lowest literature data. Low propionic acid content is generally ascribed to the peroxide—catalase treatment of milk which inhibits growth and activity of propionic bacteria (KIERMEIER *et al.*, 1968a). In Emmental manufactured from milk thus treated the ratio of acetic to propionic acid was found to be 1 : 1, while the corresponding value for cheese prepared in parallel from pasteurized milk was 1 : 2.2 (KIERMEIER *et al.*, 1968a). No increase in butyric acid content was reported as a consequence of peroxide—catalase treatment of milk (JAGER, 1967). The high butyric acid content as found in this study might, perhaps, be connected with silage feeding (MAYR, 1969).

The type of rennet used in cheese-making has a distinct effect both on content and on quantitative distribution of free fatty acids. No qualitative differences in C_2—C_{16} fatty acid composition were found between the cheeses manufactured with *CR* and *MR*, resp., if the latter preparation was applied in the lowest possible concentration (KISS *et al.*, 1973, 1974), advantageous also from the point of view of sensory characteristics. With higher enzyme concentrations various qualitative differences in fatty acid composition were found as compared to the control cheeses, however, these results need further corroboration.

The effect of the microbial rennet on the *FFA* content and distribution was found to be different with various cheese types: in Emmental cheese *FFA* content increased 4-fold as compared to the control batches, whereas in Trappist it decreased by about 50%. In "Tea"-cheese the decrease was less marked, while in "Pálpusztai" and White cheese no significant differences were observed between the batches manufactured with *CR* and *MR*, resp.

As to the effect of *MR* on the quantitative distribution of free fatty acids, the findings concerning Emmental cheese — as mentioned in the paper cited (VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974) — are considered important: applying the microbial preparation shifted the ratio of acetic and propionic acids to 1 : 2.9 which corresponds to the majority of literature data published on the subject. At the same time a decrease in relative butyric acid content

was achieved, too (the ratio of propionic and butyric acids resulted to be 1 : 0.22).

The changes induced by the use of *MR* are less marked in semi-hard and soft cheeses manufactured from cow's milk. In Trappist the relative decanoic acid content significantly decreased on applying the microbial preparation. Since a "pungent-hot" taste is attributed to this compound in dairy products (DAVIS, 1966), this decrease may be advantageous from the point of view of sensory qualities. In "Tea"-cheese, similarly to Trappist, a decrease in decanoic acid content was brought about by *MR*, accompanied by a simultaneous decrease in the respective values of hexanoic and octanoic acids. The use of the microbial preparation had an opposite effect on butyric acid formation in the two soft cheese types investigated as prepared from cow's milk: while it increased the relative amount of this compound in "Tea"-cheese, it acted contrary in "Pálpusztai".

White cheese, a soft cheese manufactured from ewe's milk, proved less sensitive to the renneting agent: no changes in *FFA* content or composition were brought about by *MR*.

The quantitative differences as established by objective methods of analysis were not reflected in the sensory characteristics of the cheeses as will be shown in detail in a forthcoming paper.

*

The authors are indebted to the respective leaders and staffs of the cheese plants mentioned in para. 1.1. of the paper for helpful assistance in the cheese-making experiments as well as to Mrs. VALÉRIA KRISTOFORY for assiduous laboratory work.

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A kiadásért felel az Akadémiai Kiadó igazgatója. Műszaki szerkesztő: Botyánszky Pál
A kézirat nyomdába érkezett: 1975. III. 6. — Terjedelem: 10,5 (A/5) ív 67 ábra, (4 szines)

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21. VIII. 1975

ACTA ALIMENTARIA

ACADEMIAE SCIENTIARUM HUNGARICAE

VOL. 4 NUMBER 4

AKADÉMIAI KIADÓ, BUDAPEST

1975

ACTA ALIMENTARIA

ACADEMIAE SCIENTIARUM HUNGARICAE

A QUARTERLY OF THE COMMITTEE ON FOOD SCIENCE
OF THE HUNGARIAN ACADEMY OF SCIENCES

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Address of the Editorial Office:

Central Food Research Institute
H-1022 Budapest, Herman Ottó út 15. Hungary

Acta Alimentaria is a quarterly publishing original papers on food science in English. The main subjects covered are: physics, physical chemistry, chemistry, analysis, biology, microbiology, enzymology, engineering, instrumentation, automation and economics of foods and food production.

Acta Alimentaria is published in quarterly issues comprising about 400 pages per year.
Subscription price: $ 32.00 per volume

Distributor:

KULTURA
Hungarian Trading Co. for Books and Newspapers
Budapest 62, P.O. Box 149, Hungary
or its representatives abroad, listed on p. 3 of the cover.

Acta Alimentaria is published by

AKADÉMIAI KIADÓ
Publishing House of the Hungarian Academy of Sciences
Budapest 502, P.O. Box 24, Hungary

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Acta Alimentaria, Vol. 4 (4), pp. 331—340 (1975)

CHANGES IN THE FATTY ACID COMPOSITION OF RAPE-SEED OIL AND A MIXTURE OF RAPE-SEED OIL AND WHEAT GERM OIL, DUE TO FRYING*

R. KURKELA and M. PETRÓ-TURZA

(Received January 21, 1974)

The influence of the addition of wheat germ oil on the heat degradation of frying oil due to heating was studied by determining chromatographically the fatty acid composition of rape-seed oil and of a mixture of rape-seed oil and wheat germ oil (10%) when heated under conditions similar to frying. It was found that the decrease of oleic, linoleic and linolenic acids due to heating was effectively retarded by the presence of wheat germ oil. Loss of oleic acid was completely retarded, that of linoleic acid diminished to one third and that of linolenic acid to half of the loss without wheat germ oil during 88 h of heating. It was also established that the presence of wheat germ oil improved the heat resistance of erucic acid, but the exact quantitative evaluation needs an improvement of the method to determine erucic acid more accurately.

The rapid deterioration of frying oils is both an economic and a nutritional problem of great importance since the popularity of fried foods is continuously increasing. Hydrolysis, oxidation, degradation and polymerization of oil at elevated temperatures causes the oil to foam, the colour to darken, off-flavours to develop and the nutritional value of the oil to decrease. Many antioxidants, particularly those occurring naturally, such as tocopherols, become less effective even inactive at the conditions of deep frying (CHANG & MONE, 1960). Some additives, as polysiloxanes (BABAYAN, 1957; DOBSON *et al.*, 1969), phosphoric acid (AKKEREN, 1966) and α-sitosterol (CHANG & MONE, 1960), are used to retard the foaming tendency. The mechanism of antifoaming activity is not completely clear. Polysiloxanes probably prevent the oxidation of fat (PADLEY *et al.*, 1970), phosphoric acid delays the polymerization (AKKEREN, 1966), and the isofucosterol structure seems to be effective in protecting the oil from oxidative polymerization (SIMS *et al.*, 1972). In an earlier study it was established that wheat germ oil mixed as such with frying oil retarded the heat-induced physical changes (colour and viscosity) of frying oil (KURKELA & KAURALA, 1971). For this reason the fatty acid composition of the frying oils after heating in the absence and presence of wheat germ oil were determined.

* Contribution in EKT series 361, University of Helsinki, 1975.

1. Materials and methods

1.1. Materials

Frying oils. Rape-seed oil *(Kultasula, Kasviöljy Oy, Raisio)* was used as reference frying oil. Wheat germ oil was extracted with petroleum ether (40—65 °C) from wheat germs and the solvent was evaporated in a vacuum evaporator. The oil was degummed and neutralized (ANDERSEN, 1962). A mixture of rape-seed oil and wheat germ oil (9 : 1) was used as the other frying oil sample. Conditions of deep fat frying were imitated by conducting water vapour through the oil during heating (190±5 °C). Samples were taken every eight hours until heating had continued for 88 h in the case of rape-seed oil, and for 100 h in the case of the mixture. The characteristics of the frying oils in the fresh state and after 88 h heating are shown in Table 1.

Table 1

Physical and chemical characteristics of frying oils in the fresh state and after 88 h heating

	Fresh		After 88 h heating	
	Rape-seed oil	Mixture*	Rape-seed oil	Mixture*
Viscosity cP	52.5	52.5	715.0	122.5
Refr. ind.	1.4740	1.4744	1.4805	1.4762
FFA	0.22	0.22	2.8	2.4
I. V.	106.1	108.8	79.8	96.8

* Rape-seed oil and wheat germ oil (9 : 1)

1.2. Reagents

— 0.5 *N* NaOH solution in methyl alcohol

— BF_3 reagent: 125 g of BF_3 dissolved in 1 litre methyl alcohol. Preparation: 1 litre of methyl alcohol was measured into a dark reagent bottle and cooled in ice, BF_3 was introduced from the cylinder through a control valve, and the cautious introduction of the gas was continued until the desired weight was obtained. The reagent was stored in the refrigerator.

— saturated solution of NaCl

— petroleum ether

— anhydrous Na_2SO_4

— n-hexane

All reagents were MERCK's *(pro anal.)* products.

1.3. Saponification and methylation of the oils

About 150 mg of oil were weighed on an analytical balance into an *Erlenmeyer* flask with ground stopper. Four ml of 0.5 *N* NaOH solution were added and brought to the boil in a water bath of 70—75 °C under reflux and retained at this temperature for 30 min. Five ml of the BF_3 reagent solution were added and the reaction mixture was boiled for a further 10 min. After cooling, the reaction mixture was transferred to a separatory funnel with 20 ml petroleum ether. Twenty ml of saturated NaCl solution was added and the funnel was shaken thoroughly for 2 min. After the separation of the phases the petroleum ether phase was filtered through anhydrous Na_2SO_4 in an *Erlenmeyer* flask with ground stopper. After repeated washing with petroleum ether and addition of anhydrous Na_2SO_4 the extract was kept in the refrigerator overnight. Next day the petroleum ether extract was filtered into a vessel of known weight. The solvent was evaporated in nitrogen stream under mild heating. The residue was weighed and dissolved in n-hexane to 16% (w/w). The hexane solution of fatty acid methyl esters thus obtained was used for separation by gas chromatography. Thin-layer chromatography was used to control whether saponification and methylation had been carried out quantitatively (METCALFE *et al.*, 1966).

1.4. Gas chromatography of fatty acid methyl esters

Gas chromatography: PYE UNICAM series 104 *Chromatograph Heated Dual Flame Ionization Detector, Programmed Chromatograph Model 64.*

- Column: 3 m, 1/8 inch diameter stainless steel column, packed with 15% diethyl glycol succinate (DEGS) on Chromosorb W (60—80 mesh)
- Carrier gas velocity: 24 ml min^{-1}
- Attenuation: 2×10^4
- Paper speed: 4 mm min^{-1}
- Injection temperature: 200 °C
- Sample size: 0.25—0.5 μl

The temperature program was determined experimentally. For this purpose a standard fatty acid methyl ester mixture (HORMEL INSTITUTE) was used. The following program proved to be the most suitable: Initial temperature: 100 °C, maintained for 5 min; temperature was increased to 185 °C at a rate of 12 °C per minute and maintained at this temperature while every peak was being eluted. A chromatogram of a sample was obtained in about 60—70 min. The peak areas were measured by planimetry. Quantitative determinations were carried out by using pentadecanoic acid as an internal standard. Six peaks were identified by using HORMEL standard mixture containing palmitic, stearic, oleic, linoleic, linoleic and erucic acids.

2. Results and discussion

Gas chromatograms were made from the samples of both frying oils in the fresh state and after every 8 h heating. Besides the peak of the solvent eluted at first, the chromatograms of both fresh oils contained 18 peaks each.

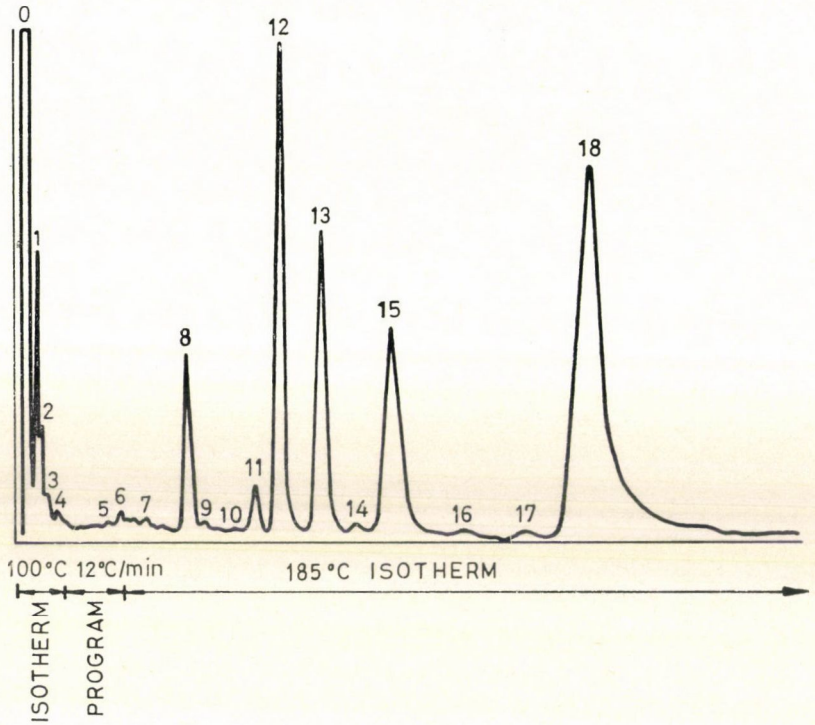

Fig. 1. Chromatogram of fatty acid methyl esters obtained from rape-seed oil sample being heat-treated for 48 h. Column packed with: 15% diethyl glycol succinate (DEGS) on Chromosorb W (60—80 mesh). Carrier gas velocity: 24 ml min^{-1}. Attenuation: $2 \cdot 10^4$. Injection temperature: $\sim$ 200 °C. The chromatogram run according to the temperature program indicated in the figure. The peak of the solvent eluted at first was marked by 0. From the other methyl ester peaks (18 in number) only 6 peaks, being present in greater quantity, were identified by the use of a standard methyl ester mixture. These are as follows: Peak No. 8.: palmitic acid (C_{16}); peak No. 11.: stearic acid (C_{18}); peak No. 12.: oleic acid ($C_{18=1}$); peak No. 13.: linoleic acid ($C_{18=2}$); peak No. 15.: linolenic acid ($C_{18=3}$); peak No. 18.: erucic acid ($C_{22=1}$)

The number of peaks was the same in all samples, only the ratio of peak areas differed.

The chromatogram of rape-seed oil methyl esters (oil heated 48 h) is presented in Fig. 1.

Since it is probable that palmitic acid and stearic acid, being saturated acids, are very little affected under these heating conditions, palmitic acid was taken as a reference and the peak areas of five other fatty acid methyl

esters identified were measured in relation to that of palmitic acid. The relative fatty acid contents of rape-seed oil and of the mixture of rape-seed oil and wheat germ oil thus obtained are stated in Tables 2 and 3.

Changes in the concentration of five fatty acids were plotted against the time of heating. As expected, the stearic acid content did not show any

Table 2

*Changes in the fatty acid composition of rape-seed oil upon heat treatment**

Fatty acid methyl esters / Duration of heat treatment, h	Stearic acid *f*	Oleic acid *f*	Linoleic acid *f*	Linolenic acid *f*	Erucic acid *f*
0	0.48	4.85	5.76	5.18	10.13
8	0.39	4.98	4.97	4.73	11.40
16	0.51	4.69	4.44	4.05	10.11
24	0.45	4.91	4.29	4.11	7.57
32	0.39	3.98	3.44	3.13	8.27
40	0.43	4.54	3.33	3.23	9.30
48	0.41	4.63	3.22	3.22	9.62
56	0.45	4.06	2.95	3.08	9.80
64	0.44	4.36	2.65	2.69	8.22
72	0.41	4.22	2.43	2.63	8.12
76	0.42	3.84	2.36	2.37	7.85
80	0.42	3.96	2.38	2.38	6.41
88	0.44	3.52	2.05	2.21	7.06

* The table contains the peak areas of the most important methyl esters (as separated by gas chromatography subsequent to saponification of the oil and methylation of the fatty acids thus liberated) related to the peak area of palmitic acid (ratio f). In earlier experiments the amount of palmitic acid was found unchanged upon heat treatment of the oil. The factors thus obtained may be considered indices of quantitative changes.

tendency to decrease with increase in the time of heating. The contents of the four unsaturated fatty acids did not appear to follow a linear trend and therefore the logarithmic transformation of the original data was plotted against time of heating. Lines of regression, regression equations and correlation coefficients are shown in Figs. 2 to 5. Comparison of the correlation coefficients of linear regression and those of the logarithmic transformation showed that the latter approximation was better for linoleic acid and linolenic acids. In the

Table 3

*Changes in the fatty acid composition of a mixture of rape-seed oil and 10% wheat germ oil upon heat treatment**

Duration of heat treatment, h \ Fatty acid methyl esters	Stearic acid *f*	Oleic acid *f*	Linoleic acid *f*	Linolenic acid *f*	Erucic acid *f*
0	0.40	2.79	3.83	3.07	4.88
8	0.22	3.08	4.06	3.05	6.21
16	0.30	3.45	4.68	3.35	5.05
24	0.23	2.88	3.76	2.90	5.12
32	0.29	3.26	4.28	3.23	6.74
40	0.27	3.16	3.87	2.91	4.72
48	0.28	3.37	3.87	3.08	6.86
56	0.39	3.43	3.97	3.05	6.11
64	0.30	2.91	3.41	2.74	5.96
72	0.28	3.56	3.91	3.01	6.93
76	0.26	3.56	3.86	3.02	7.17
80	0.26	3.10	3.90	2.68	5.00
88	0.28	3.05	3.38	2.56	5.93
100	0.29	3.54	3.42	2.70	6.35

* For legend see Table 2

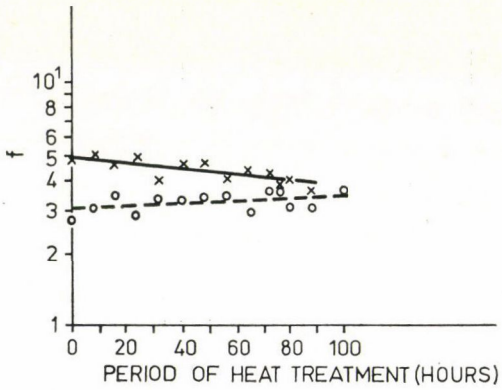

Fig. 2. Changes in oleic acid content upon heat treatment as referred to the quantity of palmitic acid. x ——— x rape-seed oil (R), o - - - - - - o rape-seed oil + 10% wheat germ oil (K). h = period of heat treatment, h. f = factor (peak area of oleic acid divided by the peak area of palmitic acid). Equations of regression lines and the correlation coefficients: $\lg f_R = 0.6984 - 0.0013\,h$, $r_R = -0.8327$; $\lg f_K = 0.4816 + 0.0005\,h$, $r_K = 0.4388$. Results of t-tests regarding regression coefficients: $t_a = 13.7 > t_{0.001} = 3.5$; $t_b = 4.5 > t_{0.001} = 3.5$. t_a = is the t-test referring to the axial sections, and it proves that axial sections of the two straight lines significantly differ from each other. t_b = is the t-test referring to the slope of the straight lines, and its significant difference proves that the two slopes are different

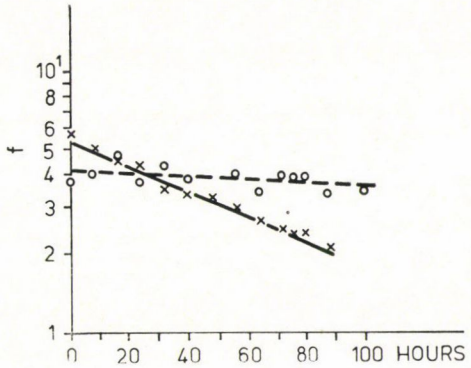

Fig. 3. Changes in linoleic acid content upon heat treatment as referred to the quantity of palmitic acid. x ——— x rape-seed oil (R), o - - - - - - o rape-seed oil + 10% wheat germ oil (K). h = period of heat treatment, h. f = factor (peak area of linoleic acid divided by the peak area of palmitic acid). Equations of regression lines and the correlation coefficients: $\lg f_R = 0.7320 - 0.0048\,h$, $r_R = -0.9909$; $\lg f_K = 0.6230 - 0.0007\,h$, $r_K = -0.6004$. Results of t-tests regarding regression coefficients: $t_a = 5.8 > t_{0.001} = 3.5$; $t_b = 9.3 > t_{0.001} = 3.5$

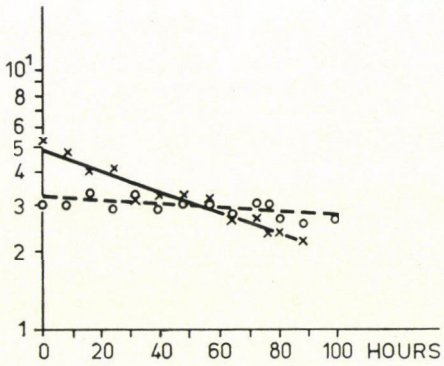

Fig. 4. Changes in linolenic acid content upon heat treatment as referred to the quantity of palmitic acid. x ——— x rape-seed oil (R), o - - - - - - o rape-seed oil + 10% wheat germ oil (K). h = period of heat treatment, h. f = factor (peak area of linolenic acid divided by the peak area of palmitic acid). Equations of regression lines and the correlation coefficients: $\lg f_R = 0.6892 - 0.0040\,h$, $r_R = -0.9766$; $\lg f_K = 0.5055 - 0.0007\,h$, $r_K = -0.6900$. Results of t-tests regarding the regression coefficients: $t_a = 14.0 > t_{0.001} = 3.5$; $t_b = 9.6 > t_{0.001} = 3.5$

case of oleic acid the approximations were similar, and linear regression was somewhat better fitted for erucic acid.

The variations of the stearic acid and palmitic acid ratio are within the limits of reproducibility of the method (10%), showing that stearic acid and palmitic acid behaved similarly under heating. Quantitative determination of stearic and palmitic acids made from both the fresh oils and from the oils after the longest time of heating (Table 4) show that the contents

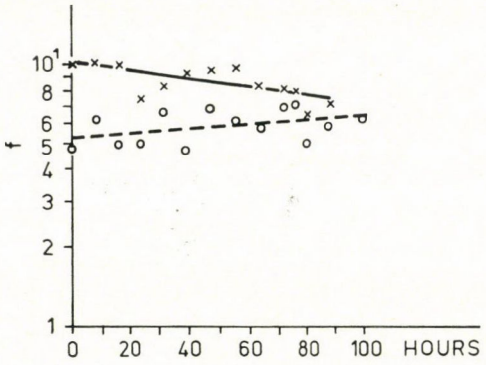

Fig. 5. Changes in erucic acid content upon heat treatment as referred to the quantity of palmitic acid. x ——— x rape-seed oil (R), o - - - - - o rape-seed oil + 10% wheat germ oil (K). h = period of heat treatment, h. f = factor (peak area of erucic acid divided by the peak area of palmitic acid). Equations of regression lines and the correlation coefficients: $\lg f_R = 1.0169 - 0.0017\,h$, $r_R = -0.6993$; $\lg f_K = 0.7281 - 0.0008\,h$, $r_K = 0.4057$. Results of t-tests regarding the regression coefficients: $t_a = 8.1$, $t_{0.001} = 3.5$, $t_b = 2.7$, $t_{0.01} = 2.5$

of these acids are unchanged in all samples even after the longest time of heating. A decrease of up to 8.8% is within the limits of accuracy of the method.

The quantitative values of oleic acid, linoleic acid and linolenic acid (Table 4) confirm the earlier observations concerning these acids showing that the presence of wheat germ oil retarded the degradation of these acids very effectively. There was no decrease of oleic acid during 100 h heating of the rape-seed oil and wheat germ oil mixture, while a 20% decrease occurred

Table 4

Quantitative changes of fatty acids in oils after heat treatment

	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Erucic acid
	g in 100 g oil					
Fresh rape-seed oil	3.4	1.5	13.6	17.2	17.4	36.6
Rape-seed oil treated for 88 h	3.1	1.4	10.9	6.1	6.7	22.6
Reduction as % of fresh oil	8.8	6.8	19.4	64.6	61.3	38.4
Fresh oil mixture*	4.4	1.1	12.5	17.2	13.7	26.8
Oil mixture treated for 100 h	4.2	1.1	12.4	13.8	9.8	19.1
Reduction as % of fresh oil	4.5	0	0.8	19.8	28.5	28.7

* Mixture of rape-seed oil and 10% wheat germ oil

Quantitative evaluation of the fatty acids was performed by adding a known amount of penta-decanoic acid standard to the oil prior to saponification and relating the peak area of this to those of the other fatty acids.

in the pure rape-seed oil during 88 h heating. In the mixed oil the decrease of linoleic acid was a third of that in the pure oil, and the decrease of linolenic acid in the mixture was half of that in the pure oil. Retardation of erucic acid degradation was also shown by quantitative determinations (Table 4). However, concerning erucic acid there is a discrepancy in the results obtained with palmitic acid as reference, and those obtained with added pentadecanoic acid. As shown in Fig. 5, the erucic acid content of mixed frying oil did not decrease due to heating; on the contrary, the erucic acid content relative to that of palmitic acid increased. Quantitative determinations showed, however, about 30% decrease after 100 h heating. This is probably due to the fact that the method was less accurate for erucic acid than for the others. Erucic acid is the last to leave the column, and the peak was broad and often asymmetrical so that it was difficult to evaluate its area with the same accuracy as the areas of acids leaving earlier. The unambiguous evaluation of the behaviour of erucic acid requires further investigations.

3. Conclusions

The changes in fatty acid composition caused by heating of rape-seed oil were compared with those of a mixture of rape-seed oil and wheat germ oil (9 : 1). The results showed that the decrease of the unsaturated fatty acids as oleic, linoleic, linolenic and erucic acids due to heating was effectively retarded by the presence of wheat germ oil in the frying oil. Loss of oleic acid was completely inhibited, the loss of linoleic acid diminished to one third, and that of linolenic acid decreased to half of the original loss. It was proved that erucic acid was more heat resistant in the presence of wheat germ oil, but the exact quantitative evaluation of GLC data needs further investigations.

It is obvious that some constituents of wheat germ oil retard the oxidation-polymerization reactions in the oils due to heating. These findings are in accordance with earlier observations concerning the physical changes of frying oils.

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Acta Alimentaria, Vol. 4 (4), pp. 341–353 (1975)

WEIGHTING OF QUALITY CHARACTERISTICS IN THE SENSORY EVALUATION OF FOODS BY SCORING*

R. LÁSZTITY and F. ÖRSI

(Received August 1, 1974)

The fundamental problems related to systems of sensory evaluation by scoring are the following:
— selection of the appropriate quality characteristics,
— setting up the scoring scale,
— weighting the individual quality characteristics.
The last of the problems is of particular importance. A prerequisite of developing the mathematical model of evaluation by scoring is the determination of the weighting factors. This may be achieved by utilizing discriminance analysis.
A scoring system based on values obtained by discriminance analysis appears to be suitable for the development of an evaluation system more reliable and exact than earlier ones, as shown in tests carried out with bottled peaches and morello cherries, as well as canned peas.

Beside their nutrient and active agent content the value of foodstuffs is highly influenced by their sensory value, thus in judging the quality of foods the determination of their sensory value is of extreme importance. At present organoleptic qualities are estimated almost exclusively by sensory methods, since the instrumental evaluation of sensory characteristics was successful only in certain fields. For instance the textural quality of some foodstuffs was successfully established by the measurement of their rheological properties. In other foods the colour is tested by instrumental measurement (photometer, tintometer). Extensive research work was required to elucidate the correlations and the instruments used are calibrated against our sense organs.

To evaluate the sensory value of foods in the standards of different countries different scoring systems are specified. Difference methods (paired comparison, duo-trio test, triangle test) may be used only if the required quality is established in a standard sample and evaluation is carried out by direct comparison. Difference techniques are suitable for the numerical evaluation of individual properties in case several grades may be applied to evaluate the trend of differences between samples, that is, to determine which sample

* Paper read at the international conference organized by the POLISH ACADEMY OF SCIENCES, Pulawy, on "Sensory analysis: Proceedings in sensory evaluation of odour and taste".

and to what extent is preferable to the other. However, this technique may not be extended over more than one property at a time, thus it provides significantly less information than scoring as specified in most quality standards.

In order to develop a scoring system of scientific claim the settling of three basic problems is required:

1. Selection of appropriate quality characteristics;
2. Fixing the scoring scale;
3. Weighting of the individual quality characteristics.

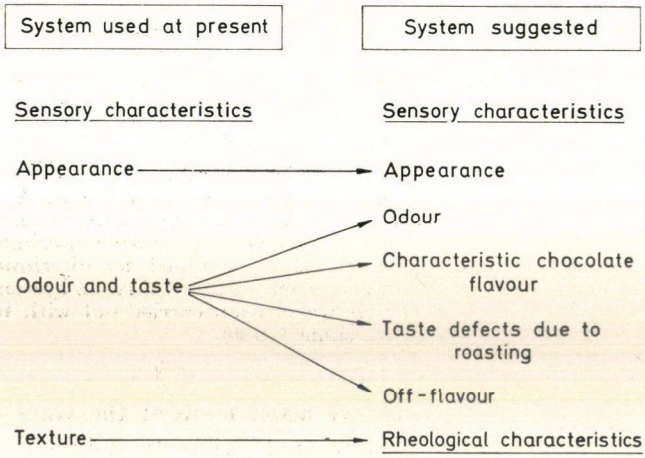

Fig. 1. Suggested changes of sensory characteristics in chocolate grading

The selection of a quality characteristic having an important bearing on the sensory quality of a food has been the subject of thorough investigations. However, in relation to certain foods, practical experience has shown the necessity of reexamination. It seems desirable to abandon scoring of properties to the measurement of which instruments are available, or in other cases to adapt the scoring of individual taste factors instead of overall scoring of the taste. The latter is particularly indicated if samples of complex taste character have to be judged and it would be extremely difficult to express various taste defects by a single characteristic.

For instance in evaluating chocolate the texture could be measured with a penetrometer or by some other rheological instrument, while instead of the combined evaluation of taste and odour (HUNGARIAN STANDARD, 1966) the characteristic chocolate odour, taste defects due to defective roasting and off-flavours could be separately scored.

The system used hitherto and the suggested method are shown in Fig. 1.

The fixing of a scoring scale is an intricate task. The standards of different countries specify different scoring scales. In most of them the weight of the

ndividual characteristics is expressed by the difference in the length of the scale. The difference in the scale length however, as expressed earlier in mathematical terms (ÖRSI, 1972), highly affects the distribution of scores. To illustrate the above in Fig. 2 the frequency distribution of the scores for taste, colour and odour as well as of the total score of bottled sour cherries is given.

The density function of the normal probability distribution of equal average value and standard deviation is also illustrated in the figure. As can

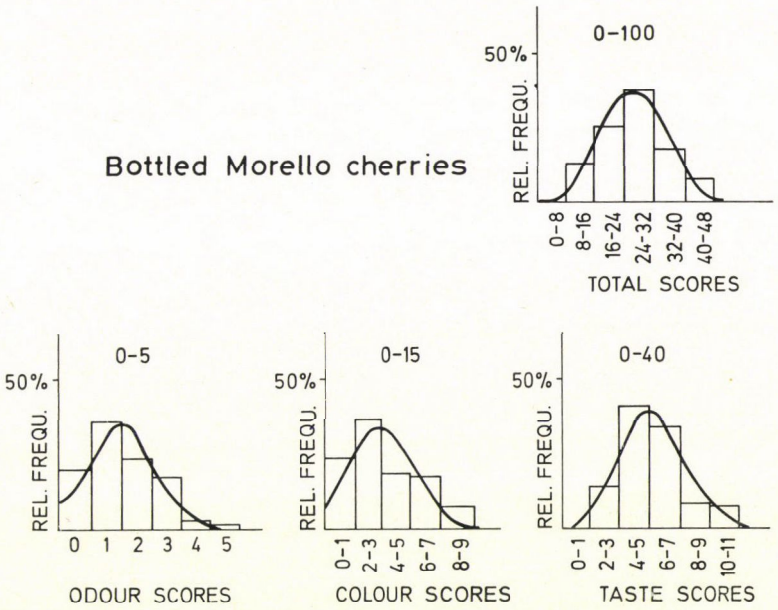

Fig. 2. Distribution of scores in the sensory evaluation of bottled sour cherry in case of different scale lengths

be seen, the distribution of scores over the scales of 0—5, 0—15, 0—40 and 0—100 approximates the normal distribution the more closely the longer the scale is. It is evident that deviations from the normal distribution appear also in the evaluation of the results. If statistical methods based on normal distribution are intended to be used, longer scales are necessary and they should be of the same length for all the characteristics. The weighting of individual characteristics is preferably done by multiplication with weighting factors.

The standards prepared in the GDR (GERMAN STANDARD, 1972) are of advanced character because a uniform 5-point scale is used for judging every characteristic and the results are multiplied by factors to give each characteristic the appropriate weight.

In developing a scoring system the proper weighting of the characteristics is the crucial point. Weighting of the characteristics enables the individual properties to participate according to their respective importance in the total score and in the evaluation of quality.

This study was aimed at the investigation of some problems related to food evaluation by scoring and in particular of those of weighting.

1. Materials and methods

1.1. Samples

The samples used for sensory evaluation were obtained from the NAGYKŐRÖS CANNING FACTORY. Since samples of different quality were needed, some of them were taken directly from the processing line, others from storage.

Three products were tested: bottled peaches, sour cherries and peas. They were contained in jars of 1 kilogram capacity and the former two were pasteurized in open water baths, the peas were sterilized in a retort under pressure.

1.2. Sensory evaluation

The sensory evaluation was carried out by a panel of 8 to 10 trained members in accordance with standard specifications (HUNGARIAN STANDARD, 1952).

1.3. Discriminance analysis

The results of the sensory evaluations were ranked, taking into account total scores and taste scores, in quality categories as specified in the standard. Calculations were carried out at the UNIVERSITY COMPUTER CENTER with a *Razdan* 3 type computer. The program was prepared in the *Algol* programing language. The data-tape contained the number of quality grades, the number of selected characteristics, the number of data per grade and the data themselves. In case of 3 grades, 5 characteristics and 100 data the time required for processing was about 5 min.

Results included the coefficients of the discriminance function, the result of variance analysis used to decide about the suitability of the discriminance equation and the new total values derived by the discriminance equation.

2. Results and conclusions

The use of scoring for evaluation is advantageous because it permits of evaluating several characteristics simultaneously.

Evidently, the more characteristics are used in establishing the quality grade the sounder is the decision. However, increasing the number of character-

istics involves problems. In Fig. 3 a quality field is seen, which was determined on the basis of two characteristics. It is clear that in the hatched fields, to which the "good" or "bad" values of both characteristics belong, the sample will be of grade I or II. However, to reach a decision in the white fields is not unambiguous.

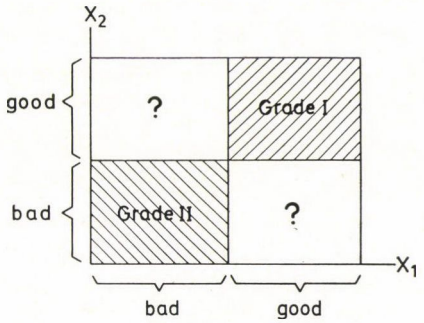

Fig. 3. Quality field determined by two characteristics (X_1 and X_2)

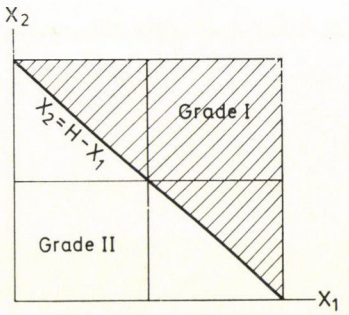

Fig. 4. Quality field showing the conditional equation

The method used at present, based on the total score, solves the problem of reaching decision by dividing the quality field into two by Equ. 1:

$$H = X_1 + X_2 \qquad \text{(Equ. 1)}$$

where X_1 and X_2 are the values of the two characteristics and H is the boundary value of the total score dividing the two grades.

By using the equation, the field considered problematic as regards decision, is divided into two, as seen in the quality field in Fig. 4. The section containing the "better" characteristic values is classified as belonging to grade I, while the section containing the lower values to grade II.

If the two characteristics are of different weights this will appear in the equation in the form of multiplying factors. Such weighted characteristics are present in Equ. 2:

$$H = B_1X_1 + B_2X_2 \qquad \text{(Equ. 2)}$$

where B_1 and B_2 are factors determining the weight of the respective characteristics.

By transforming Equ. 2 the following expression is obtained

$$X_2 = \frac{H}{B_2} - \frac{B_1}{B_2} X_1 \qquad \text{(Equ. 3)}$$

If $B_2 > B_1$, characteristic B_2 is of greater weight and the slope of the line is below 45°. In this case, as seen in Fig. 5, obviously the second characteristic is weighted lower, therefore on the basis of this characteristic fewer samples will be classified as grade I, than without weighting.

If there are more than two characteristics the quality field is illustrated by a multi-dimensional space in which the equation (Equ. 4) used to calculate the total score forms a multi-dimensional plane and this will divide the quality field into quality grades.

$$H = B_1X_1 + B_2X_2 + \ldots + B_nX_n \qquad \text{(Equ. 4)}$$

In the scoring systems used at present accounting for the weight of individual characteristics is done empirically, in a subjective way or, at best,

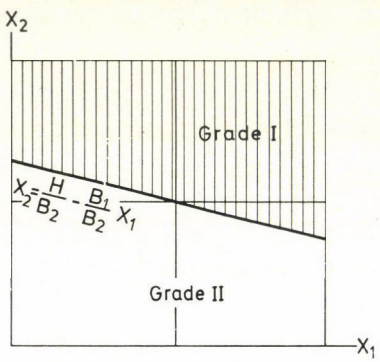

Fig. 5. Quality field showing the conditional equation in case of weighted characteristics

on the basis of calculation technical considerations. However, the multiplying factors, apart from determining the weight of individual characteristics, are important from the point of view of grading the product.

Fig. 6 gives an illustration of the density function of the total score of products belonging into grades I and II. The products the total score of which falls in the hatched field may not be unambiguously classified as grade

I or grade II, without making some mistake. Therefore it is desirable to reduce the overlapping area. This may be achieved in two ways, either by increasing the difference

$$d = \bar{X}_{II} - \bar{X}_{I}, \text{ or}$$

by reducing the s value of deviations.

The weighting factors in the equation used for the calculation of the total score may be determined according to the discriminance analysis method of FISHER (1936) and utilizing earlier analytical results, so as to satisfy maximally these two conditions.

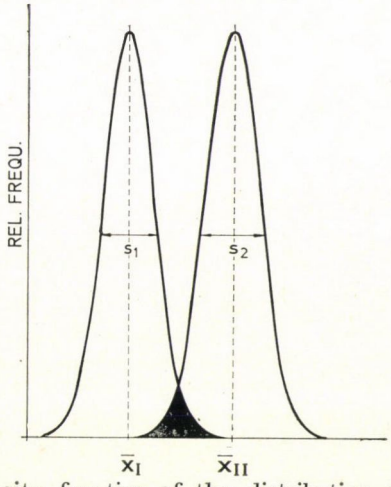

Fig. 6. Density function of the distribution of total score

Deduce a quotient Q by using the difference in the averages and the sum of variances according to Equ. 5:

$$Q = \frac{d^2}{s_I^2 + s_{II}^2} \qquad \text{(Equ. 5)}$$

where $d = \bar{X}_I - \bar{X}_{II}$ is the difference between the average values and s_I and s_{II} mark the deviations.

The Q value thus obtained is the higher the smaller the overlapping of the two distributions. Since the Q value is a function of B weighting factors, the latter have to be selected to give Q the highest value possible. To achieve this, Q function is differenciated according to $B_1, B_2, B_3 \ldots B_n$ and the quotients are reduced to zero or in other words the place of the maxima of the Q quotients belonging to each B value is determined. The system consisting of n conditional equations ends in Equ. 6:

$$\begin{aligned} q_{11}B_1 + q_{12}B_2 + \ldots . + q_{1n}B_n &= d_1 \\ q_{21}B_1 + \ldots\ldots\ldots\ldots + q_{2n}B_n &= d_2 \\ &\cdot \\ &\cdot \\ &\cdot \\ q_{n1}B_1 + \ldots\ldots\ldots\ldots + q_{nn}B_n &= d_n \end{aligned} \qquad \text{(Equ. 6)}$$

where $B_1 \ldots B_n$ are the weighting factors sought for, $d_1 \ldots d_n$ the difference between the average values of the two characteristics in the two quality grades.

q_{ij} values are calculated by Equ. 7:

$$q_{ij} = \sum_{m=1}^{k_I} X_{iI}(m)\, X_{jI}(m) + \sum_{m=1}^{k_{II}} X_{iII}(m)\, X_{jII}(m) - \frac{\sum_{m=1}^{k_I} X_{iI}(m) \sum_{m=1}^{k_I} X_{jI}(m)}{k_I} - \frac{\sum_{m=1}^{k_{II}} X_{iII}(m) \sum_{m=1}^{k_{II}} X_{jII}(m)}{k_{II}} \qquad \text{(Equ. 7)}$$

where:

k_I and k_{II} are the resp. numbers of analytical results in grades I and II;
X_{iI} = the values of i quality characteristic in grade I;
X_{jII} = the values of j quality characteristic in grade II;
m = index of the samples.

The B_i values derived by solving the simultaneous equations are the multi pliers in the equation used for calculating the total scores. Since no constant member is included in the equation the B_i values may be multiplied by any constant. This is useful from the point of view of practical application, because the B_i values, when fractions, may be transformed into whole numbers or the total score may be adjusted to the desired length of the scale.

The application of the method is illustrated on the basis of the results obtained by the standard test of bottled peaches, manufactured in the NAGYKŐRÖS CANNING FACTORY. The 6 quality characteristics by which the sensory value is established (HUNGARIAN STANDARD, 1958) are: colour, odour, taste, texture, shape and defects of the raw material.

These characteristics were evaluated as described in para. 1.2. in 140 samples. The distribution of the scores deducted for defects is shown in Figs. 7 and 8.

The frequency distribution of scores deducted for defects in colour, odour and taste is shown in Fig. 7. Though the distribution differs for products of grade I and grade II, a line unambiguously separating products of grade I and grade II cannot be drawn.

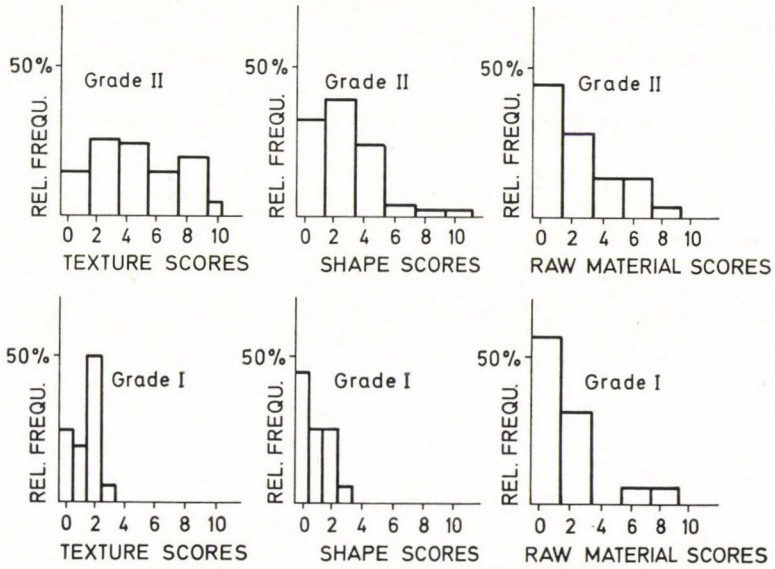

Fig. 7. Distribution of the scores for colour, odour and taste of bottled peaches

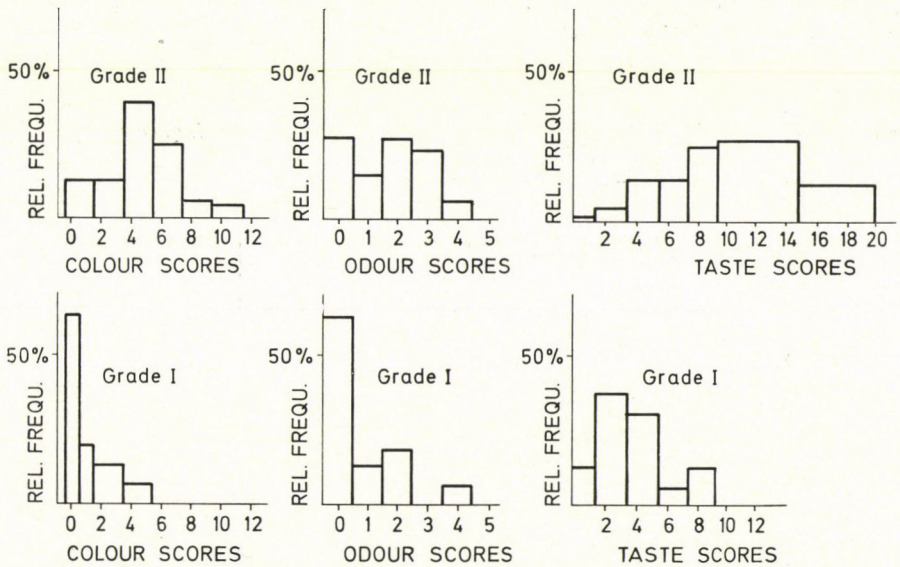

Fig. 8. Distribution of the scores for texture, shape and defects of raw material in bottled peaches

The frequency distribution of scores deducted for defects in shape and raw material, as shown in Fig. 8, presents a similar picture.

Instead of simply calculating the total scores, a discriminance equation (Equ. 8) was established:

$$X = B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_5X_5 + B_6X_6 \qquad \text{(Equ. 8)}$$

where

B_1, B_2, B_3, B_4, B_5, B_6 are the weighting factors to be calculated,
X_1 is the score deducted for colour defect,
X_2 is the score deducted for odour defect,
X_3 is the score deducted for taste defect,
X_4 is the score deducted for defects in texture,
X_5 is the score deducted for defects in shape,
X_6 is the score deducted for defect in raw material,
X is the index number used for classification.

The new evaluating system developed on the basis of the coefficients of the discriminance function calculated according to para 1.4, compared to the standard method, is shown in Table 1.

In comparison to the original standard method various modifications seem necessary in the system based on the discriminance equation. The weight of colour has to be increased substantially, that of the texture slightly. The weight of taste and of defects of the raw material has to be reduced substantially while that of shape slightly. In case of the products investigated the role of these characteristics differs from the original conception.

Table 1

The standard method and the new scoring system based on discriminance analysis for the evaluation of bottled peaches

Quality characteristic	Maximum detractable score	
	According to the standard method	In the new system
Colour	15	35
Odour	5	5
Taste	40	29
Texture	10	11
Shape	10	9
Defects of the raw material	10	1
Value dividing grade I and II	82	83

The distribution in grade I and grade II of the total scores obtained by weighting the results of discriminance analysis is shown in Fig. 9.

Discriminance analysis has shown that 5% of the products were earlier ranked wrongly because the total score as computed by the discriminance equation fell in the field beyond the dividing line.

During the last three years several products of the canning industry were tested similarly (HUNGARIAN STANDARD, 1964).

Though the data processed were of limited amount it is possible to draw some conclusions.

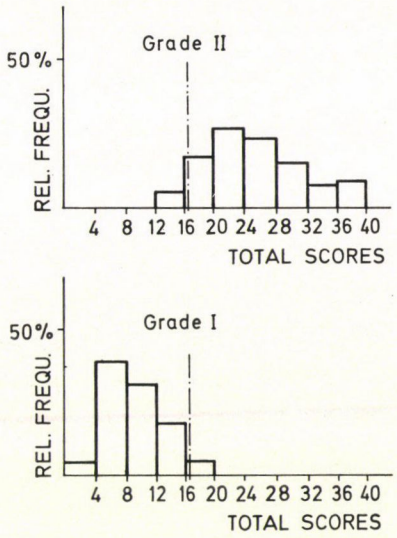

Fig. 9. Distribution of total scores of bottled peaches obtained by weighting in accordance with discriminance analysis

The first conclusion is that the method used was basically satisfactory, substantial changes are not needed. However, certain corrections seemed necessary. The system has to be adapted to certain products, even to different periods.

Table 2 lists the results of studies in relation to some products of the canning industry.

During three consecutive seasons 100 products were subjected to sensory evaluation according to para. 1.2. The results were processed by discriminance analysis in accordance with para. 1.3.

As can be seen, the deviations may be substantial in certain periods with certain products.

The amount of the processed data is not sufficient to draw further conclusions. It seems evident, however, that the deviations exposed by dis-

Table 2

Results obtained by discriminance analysis of the sensory values of products of the canning industry in three subsequent seasons

Characteristics	Standard scores	Bottled sour cherries			Bottled peaches			Green peas		
		1970	1971	1972	1970	1971	1972	1970	1971	1972
Colour	15	6	13	13	35	30	14	19	21	15
Odour	5	8	6	6	5	15	3	4	5	5
Taste	40	41	45	40	29	24	40	38	27	29
Texture	10	10	9	10	11	3	10	18	11	13
Defects of the raw material	10	14	8	9	1	12	7	8	27	20
Value dividing grade I and grade II	72	79.5	77	78	73	78	83	79	66	79

criminance analysis may be traced to technological defects and the exposure of these defects is of great importance.

Coming back to the original product, to peaches, it was established that in case of insufficient heat treatment or in the presence of more than average oxidase enzyme content they are apt to become brown. Since the colour defect was responsible for the lower value, it was obvious that the weight of this characteristic was increased by discriminance analysis.

A greater amount of data is at present under processing and it is hoped that the results obtained from the data representing a longer period will permit drawing conclusions of more general validity.

Discriminance analysis thus may be advantageously applied in the weighting of quality characteristics accounting for several properties at the same time. Though the calculations required are of great volume, with modern computers they can be carried out in a short time with great precision and thus evaluation will be more exact.

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Acta Alimentaria, Vol. 4 (4), pp. 355—365 (1975)

POLYPHENOLOXIDASE OF *SOLANUM MELONGENA* AND ITS NATURAL SUBSTRATE

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(Received October 9, 1974)

The polyphenoloxidase enzyme from brinjal *(Solanum melongena)* was isolated from the pulp by buffer extraction, purified by precipitation with ammonium sulphate and acetone-polyvinylpyrrolidone, and its properties were studied. The enzyme exhibited an optimum pH of 6.8. Its *Michaelis-Menten* constant was 1.675 m*M* for catechol. Heat inactivation of the enzyme was found to follow first order kinetics. There was 90% destruction on exposure to 50, 60 and 70 °C for 16, 7.3 and 1.4 min, respectively. The activation energy was 26.66 kcal per mole. The enzyme was active toward diphenol substrates but inactive toward monophenols. Cysteine, sodium chloride, sodium metabisulphite and sodium diethyl dithiocarbamate inhibited the activity to 49, 53, 58 and 81% levels at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-5} *M*, respectively. The activity was decreased on dialysis against cyanide by about 77% but the enzyme was reactivated by 8 μmoles of copper per 5 mg enzyme protein almost to the original value.

One phenolic compound with R_f values of 0.5 and 0.48 in *n*-butanol—acetic acid—water (4 : 1 : 5) and 2% acetic acid systems, respectively, was shown to act as a substrate for the polyphenoloxidase enzyme. The compound was characterized as a derivative of caffeic acid.

Brinjal fruit *(Solanum melongena)* when cut apart and exposed to air darkens indicating the presence of an oxidative enzyme system. This type of discolouration in fruits and vegetables is considered to be the result of aerobic oxidation of naturally occurring phenolic substances catalyzed by a copper-containing enzyme, polyphenoloxidase, although peroxidases, ascorbic acid oxidase and other oxido-reductases may also be involved (MAKOWER & SCHWIMMER, 1957). Polyphenoloxidases of a number of fruits and vegetables have been studied. The enzyme from potatoes (CLARK *et al.*, 1957) was the first to be isolated but mushrooms are a more concentrated source (PALMER, 1963). The polyphenoloxidases are present in apples, apricots, cherries, peaches and pears (JOSLYN & PONTING, 1951), bananas (PALMER, 1963), dates (MAIER & METZLER, 1965), avocado and egg-plant (KNAPP, 1965) and sweet potatoes (SCOTT & KATLAN, 1957) but are absent from citrus fruits, melon and tomatoes (JOSLYN & PONTING, 1951; PONTING, 1960). Very few reports have been published on the polyphenoloxidase of brinjal (KNAPP, 1961; KNAPP, 1965; SAKAMURA *et al.*, 1966; RHOADES & CHEN, 1968).

The enzymes oxidising phenols are known by several trivial names such as polyphenoloxidase, tyrosinase and catecholase. The systematic name for

a fairly well-defined enzyme in this group is o-diphenol : O_2 oxido reductase (E. C. 1.10.3.1). Polyphenoloxidase is claimed to catalyse the oxidation of two monohydroxy phenols, para-cresol and tyrosine, both with para-substituted-CH_2 groups, as well as of a number of ortho dihydroxy phenols. The latter include caffeic acid, catechol, chlorogenic acid, dihydroxy phenylalanine, pyrogallol, quercetin and myricetin. Flavonol glycosides do not behave as substrates for this enzyme (CHARLEY, 1972).

A major ortho dihydroxy compound widely distributed in fruits and vegetables is chlorogenic acid (SWAIN, 1962). The occurrence of chlorogenic, neochlorogenic and caffeic acids which give brown colour with purified polyphenoloxidase in egg-plant has been reported (SAKAMURA & OBATA, 1963). An active principle from the alkaline hydrolysate of purified extracts of *Solanum melongena* was identified as caffeic acid (BENIGNI *et al.*, 1958).

In the present work, the purification and properties of polyphenoloxidase and its natural substrate in brinjal have been investigated.

1. Materials and methods

A common purple variety of brinjal *(Solanum melongena)* of average size (6 cm long and 5 cm in diameter) was used in this work. The authentic phenolic compounds were obtained from SIGMA CHEMICALS COMPANY, USA. The reagents used were of analytical grade.

1.1. Purification of the enzyme

Fresh brinjal extract was prepared by grinding 100 g tissue (chilled, peeled and cut into pieces) in 400 ml of 0.005 *M* phosphate buffer pH 7. The insoluble pulp was removed by filtering through muslin cloth followed by centrifugation. The filtrate was held in the dark in an ice-chamber until used.

Fractional purification with ammonium sulphate was performed on the water extract at 4 °C. The precipitate formed at each concentration was removed by centrifugation at 5 000 *g* for 30 min, redissolved in 40 ml distilled water and dialyzed for 12 hrs at 0 °C.

For the precipitation of acetone powder, 100 g of frozen, peeled and cut brinjal tissue was blended with 300 ml of chilled acetone and 10 ml of a 20% aqueous solution of polyvinylpyrrolidone (PVP) in a Waring blender for 1 min and filtered through a Whatman No. 1 filter paper. The procedure was repeated once more and the residue was filtered to dryness. The powder was stored at 0 °C in a dry atmosphere.

The enzyme extracts were prepared by mixing the acetone powder with 200 ml of distilled water and keeping overnight at 4 °C. The slurry

was squeezed through a muslin cloth and centrifuged at 1 000 *g* for 30 min for further clarification.

Nitrogen was determined by the Nesslerization micro-*Kjeldahl* method (KOCH & MCMEEKIN, 1924).

1.2. Determination of polyphenoloxidase activity

The method of PONTING and JOSLYN (1948) slightly modified, was used to follow the activity of polyphenoloxidase on the basis of the rate of formation of coloured product from catechol used as substrate.

The assay was conducted by adding 1 ml of 0.01 *M* catechol solution at zero time to 1 ml of enzyme in 0.005 *M* Na_2HPO_4 solution in a total volume of 5 ml made up with 0.005 *M* phosphate buffer pH 6.8. The reaction mixture was shaken and the colour was read at 400 nm in a *Klett-Summerson Photoelectric Colorimeter* after 3 min. One unit of enzyme activity was defined as that causing a colour change equivalent to one *Klett* unit per min under the experimental conditions specified above.

1.3. Gel electrophoresis

Polyacrylamide gel (PAG) electrophoresis (acrylamide: 7.5%) was carried out on the enzyme fraction obtained at 40—70% saturation with ammonium sulphate. The electrophoresis conditions were the same as used by DAVIS (1964).

To detect polyphenoloxidase activity, the gels were immersed in a freshly prepared 0.01 *M* solution of catechol for 15 min at 30 °C. To identify the monophenoloxidase activity, the gels were treated with 0.01 *M* tyrosine for 15 min at 30 °C and then kept in contact with the active enzyme solution for 15 min at 30 °C.

1.4. Isolation of polyphenolic compounds

The polyphenolic compounds were extracted with methanol and ethylacetate as described by RAMASWAMY and REGE (1975) following the method employed by RIVAS and LUH (1968).

1.5. Detection of the substrate for polyphenoloxidase on the chromatogram

The polyphenolic compounds of brinjal in the ethyl acetate extract were fractionated by two-dimensional ascending chromatography on Whatman No. 1 paper (35 × 35 cm) at 25 °C using n-butanol—acetic acid—water (BAW 4 : 1 : 5) as the first solvent for 15 hrs and 2% acetic acid as the second

solvent for 3 hrs. After drying the chromatogram, the enzyme solution prepared by ammonium sulphate precipitation was sprayed on it and the chromatogram allowed to dry in the open air at 30 °C. The appearance of brown spots indicated the presence of a substrate.

1.6. Separation and purification of the substrate

The ethylacetate fraction was applied with a 100-μl pipette along a line 30 cm long at the narrow end of Whatman 3 mm paper sheet. Twenty-five such papers were developed in ascending direction with 2% acetic acid for 3 hrs and then dried in air. The band which browned on enzyme spraying was eluted with 95% ethanol from unsprayed papers and concentrated under vacuum.

1.7. R_f values and colour reactions

The concentrate was further purified by two-dimensional ascending chromatography using BAW (4 : 1 : 5) and 2% acetic acid systems as described earlier. The air-dried chromatograms were examined under ultraviolet and on exposure to ammonia vapour (SWAIN, 1953). One chromatogram was sprayed with freshly prepared $FeCl_3$—$K_3Fe(CN)_6$ reagent (KEPPLER, 1957), rinsed with 2% HCl and finally with distilled water. The blue spots of the polyphenolic compounds were detected and R_f values measured.

Four additional chromatograms were sprayed separately with diazotized p-nitroaniline (DPNA) (SWAIN, 1953), *Hoepfner* reagent (WALKER, 1962), vanillin reagent (SWAIN & HILLIS, 1959) and sodium borohydride (HOROWITZ, 1957). The colour characteristics of the spots were noted.

1.8. Absorption spectra

Ultraviolet and visible absorption spectra of the chromatographically purified compound were recorded in a *Beckman DK-2 Recording Spectrophotometer*. A 5% ethanolic aluminium chloride solution was used to study the shift in absorption peaks due to chelation of phenolic compounds containing ortho-dihydroxy configurations.

2. Results

2.1. Purification of enzymes

Purification of enzymes, in particular polyphenoloxidase, offers special problems because of the close association of the substrate as well as other phenolic compounds which are extremely difficult to separate and which

freely undergo nonenzymic oxidation causing browning. Several methods, therefore, had to be tried and conditions of experimentation adjusted in order to minimize these interferences.

When the acetone—polyvinylpyrrolidone extraction method (LOOMIS & BATTAILE, 1966) was adopted, a fairly colourless enzyme preparation was obtained. However, only about 10% of the enzyme activity was recovered. Ammonium sulphate precipitation gave recovery of 42.5% activity in the precipitate at 40—75% saturation. The specific activities of ammonium sulphate precipitate and acetone powder were found to be 2.5, and 1.9 units per mg protein, respectively, whereas the fresh extract contained 4.9 units per mg protein.

2.2. Properties of enzyme

The pH optimum of brinjal polyphenoloxidase was found to be 6.8. This value is different from the values of 5.0—5.2 and 7.0 reported by KNAPP (1965) and RHOADES and CHEN (1968), respectively.

On dialysis against 10^{-4} *M* NaCN for 12 h the activity of the enzyme preparation decreased to 23% of the original. It was observed that 8.8 μmoles of Cu^{++} per ml of enzyme solution (5 mg protein/ml) could reactivate the brinjal polyphenoloxidase activity to 98%.

Though the attempt to determine K_m value for egg-plant polyphenoloxidase activity on catechol by RHOADES and CHEN (1968) remained unsuccessful, the plot of $1/v$ *vs* $1/s$ in the present case showed a linear relationship within the range of 100 μM to 10 m*M* of catechol solution. The observed K_m value for brinjal polyphenoloxidase is 1.675 m*M*. KNAPP (1965) reported a $K_m = 4.0$ m*M* for egg-plant polyphenoloxidase activity with catechol as substrate.

2.3. Heat inactivation

The heat inactivation of brinjal polyphenoloxidase was carried out at 50, 60 and 70 °C and the results of these studies are shown in Fig. 1. A plot of the logarithm of residual activity versus heating time showed a linear relationship indicating that the heat inactivation of brinjal polyphenoloxidase follows first order kinetics. The linearity of the curve also showed that a single enzyme is operative or that if more than one enzyme are involved, all have identical thermal inactivation characteristics.

The *D* values indicate that 90% of the polyphenoloxidase is inactivated by heating for 16 min at 50 °C, 7.3 min at 60 °C or 1.4 min at 70 °C. From the rate constants for the heat inactivation of polyphenoloxidase, the activation energy was observed to be 26.66 kcal.

2.4. Substrate specificity

Even though the products of polyphenoloxidase activity on different substrates are reported to have different absorption maxima, the relative activity of this enzyme was determined in the present work by following the increase in colour intensity at 400 nm. The polyphenols to be enzymically oxidized are catechol, caffeic and chlorogenic acids and dihydroxyphenylalanine in the order of decreasing activity under the specified conditions.

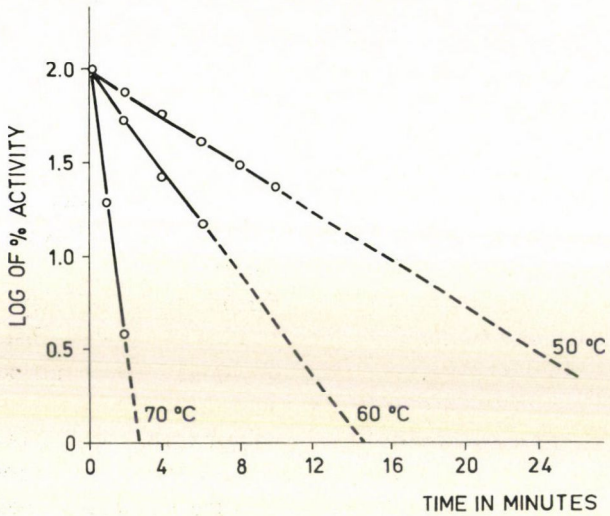

Fig. 1. Heat inactivation of brinjal polyphenoloxidase

The relative activity of the enzyme on dihydroxyphenylalanine, caffeic acid and chlorogenic acid was 30.4, 47.8 and 39.1% respectively, of that on catechol. Catechol appears to be the best substrate of the compounds tested for the crude enzyme. There was no measurable activity with monophenols tested.

2.5. Gel electrophoresis

Experiments to separate the enzyme fraction according to the electrophoretic mobility on PAG showed the existence of only one band of relatively low mobility as indicated by the brown band on catechol treatment. The fact that the electrophoretically separated pattern did not contain another protein with monophenoloxidase activity was checked by dipping the gel in tyrosine (0.01 *M*) for 15 min followed by treatment with fresh enzyme solution. No browning was noticed, showing that monophenoloxidase activity in the protein band was absent. These experiments conclusively show (1) that the

enzyme fraction moves as a single band on PAG electrophoresis; (2) that this single band corresponds only to polyphenoloxidase; and (3) that there is no monophenoloxidase activity in the enzyme fraction.

In a similar work, RHOADES and CHEN (1968) reported that tyrosinase was not the correct term for the egg-plant enzyme because monophenols were not acted upon by the same enzyme.

2.6. Inhibitors

Table 1 summarizes the effects of selected concentrations of different inhibitors on the oxidation of catechol by brinjal polyphenoloxidase. The data provide estimates of comparative activities of the agents used. Of the four inhibitors tried, sodium diethyl dithiocarbamate exerted maximum inhibition.

Table 1
Inhibition of brinjal polyphenoloxidase

Inhibitors	Concentration, M	Inhibition %	Standard deviation
1. Cysteine	10^{-3}	49	0.71
2. Sodium chloride	10^{-4}	53	0.52
3. Sodium metabisulphite	10^{-5}	57	0.40
4. Sodium diethyl dithiocarbamate	10^{-5}	81	0.35

The assay solution contained: 1 ml enzyme solution in 0.05 M phosphate buffer pH 6.8, 1 ml of inhibitors of 5×10^{-3} or 5×10^{-4} or 5×10^{-5} M concentration, 2 ml phosphate buffer pH 6.8 and 1 ml of 0.01 M catechol solution

2.7. Characterization of the substrate for polyphenoloxidase

Although eleven polyphenolics were separated by two-dimensional chromatography of the brinjal extract, only one spot developed brown colouration when sprayed with the enzyme preparation. The R_f value of this spot was found to be 0.5 and 0.48 in BAW (4 : 1 : 5) and 2% acetic acid solvent systems, respectively.

Partial purification of the substrate was carried out by one-dimensional paper chromatography with 2% acetic acid as solvent system. The compound was further purified by two-dimensional paper chromatography with BAW (4 : 1 : 5) and 2% acetic acid.

Table 2 lists the R_f values of the polyphenoloxidase substrate and the authentic polyphenolic compounds.The compound does not correspond to any of the polyphenols tried including caffeic, ferulic and chlorogenic acids. From the R_f values taken from WRIGHT and co-workers (1960) the compound with the R_f value 0.48 in the 2% acetic acid system appears to be protocatechuic acid.

Table 2
R_f values of polyphenoloxidase substrate

Compounds	R_f values			
	BAW (4 : 1 : 5)		2% HOAc	
	$\bar{x}$	s	$\bar{x}$	s
Polyphenoloxidase substrate	0.50	0.008	0.48	0.007
Standards				
Ferulic acid	0.81	0.004	0.45	0.003
Caffeic acid	0.71	0.067	0.23	0.004
Chlorogenic acid	0.61	0.005	0.70	0 003
Coumaric acid	0.89 (a)		—	
Cinnamic acid	0.90 (a)		—	
Phloroglucinol	—		0.53 (b)	
Salicylic acid	—		0.67 (b)	
m-(OH) benzoic acid	—		0.64 (b)	
p-(OH) benzoic acid	—		0.59 (b)	
p-(OH) phenyl acetic acid	—		0.76 (b)	
Protocatechuic acid	—		0.48 (b)	
3-5 dihydroxy benzoic acid	—		0.50 (b)	
Caffeic acid derivative	0.43 (c)		0.5 (c)	

(a) LEVY and ZUCKER (1960)
(b) WRIGHT *et al.* (1960)
(c) RIVAS and LUH (1968)

$\bar{x}$ = mean
s = standard devation
BAW = n-butanol-acetic acid-water

However, the colour reactions of the substrate summarized in Table 3 show a close similarity with ferulic and caffeic acids. The compound produces a reddish brown colour with diazotized p-nitroaniline whereas protocatechuic acid gives a distinct violet colouration.

Table 3
Colour reactions of the natural polyphenoloxidase substrate in brinjal

Treatment	Colour
U. V. light	Blue
U. V. + NH_3	Yellowish green
Visible light + NH_3	Yellow
$FeCl_3$—$K_3Fe(CN)_6$	Blue
DPNA*	Reddish brown
Hoepfner reagent	Yellow
Vanillin reagent	Colourless
$NaBH_4$—HCl vapour	Colourless

* DPNA = diazotized p-nitroaniline

The absorption maxima, minima and the bathochromic shift in $AlCl_3$ obtained with the compound are quite dissimilar to those obtained with the above three phenolics. The absorption spectrum shows a close similarity with chlorogenic acid (Table 4). R_f values and spectral characteristics of the compound seem to agree with those of a derivative of caffeic acid reported by RIVAS and LUH (1968).

Table 4

Spectral characteristics of the polyphenoloxidase substrate

Compounds	UV absorption		
	λ_{max} (nm)	λ_{min} (nm)	λ_{max} (nm) with $AlCl_3$
Polyphenoloxidase substrate	323, 294	264	327, 299
Standards			
Ferulic acid	320, —	—	— —
Caffeic acid	328, 298	262	— —
Chlorogenic acid	325, 295	265	328, 300
Caffeic acid derivative*	325, 295	265	328, 300

* RIVAS and LUH (1968)

3. Conclusions

According to the results, the precipitated enzyme showed a lower value of specific activity than the fresh extract of brinjal. A combination of acetone and PVP which is considered a stronger hydrogen acceptor than either acetone—polyethylene glycol or acetone alone, did not show any increase in specific activity indicating a loss in enzyme activity due to inactivation during the process of purification.

The pH optimum of brinjal polyphenoloxidase was 6.8. At alkaline pH values, autoxidation of the substrate (catechol) made it impossible to measure enzyme activity with any accuracy. At lower pH values, particularly below a pH of 3.0, enzyme activity could not be demonstrated, and subsequent raising of the pH did not restore it. The Cu of the enzyme was removed on dialysis against sodium cyanide which led to the inactivation of the enzyme. It could, however, be reactivated by adding Cu^{++}. The *Michaelis* constant, *D* values of heat inactivation and activation energy of the enzyme were also determined. The brinjal polyphenoloxidase was found to oxidize polyphenols such as catechol, caffeic and chlorogenic acids and dihydroxyphenylalanine, whereas with monophenols such as p-cresol and tyrosine, no measurable activity was

observed. On the basis of the electrophoretic mobility in PAG, it was observed that the enzyme moved as a single band showing only the polyphenoloxidase activity. The activity was inhibited by substances which form stable complexes with copper, which reduce the o-quinone before it polymerizes, and which form complexes with quinones. Sodium chloride also inhibited the enzyme by a mechanism which has not been elucidated.

The natural substrate for polyphenoloxidase enzyme present in tissues of brinjal was characterized as a derivative of caffeic acid on the basis of R_f values and colour reactions with $FeCl_3$—$K_3Fe(CN)_6$, diazotized p-nitroaniline, *Hoepfner*, sodium borohydride, and vanillin-HCl reagents, fluorescent behaviour and absorption spectra.

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Acta Alimentaria, Vol. 4 (4), pp. 367—372 (1975)

THE BREAKDOWN OF MYOFIBRILLAR PROTEINS BY SEVERE HEATING

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(Received October 25, 1974)

The changes of myofibrillar protein fraction of pork semimembranosus muscle during heat treatment were investigated. It has been found that part of the coagulated muscle protein dissolves in water after heating at high temperature (120 °C).

Measurements with the amino acid analyser confirmed the assumption that some of the muscle proteins suffer breakdown during the time of heating at 120 °C yielding free amino acids and peptides of low molecular weight.

The heating of muscle systems is an interesting field of meat research of ever-growing significance, important for the practice of meat processing in relation to nutritive value, and also to the development of flavour and aroma compounds.

Much research has been done in order to elucidate the changes in meat proteins effected by heating (HAMM, 1966; BOGNÁR, 1971; JANITZ, 1971; MARSH & FLAUMENBAUM, 1971; SAVIČ, 1972). Obviously a great deal of information has accumulated on various proteins (proteins of muscle and that of connective tissue) but the findings are restricted mainly to changes observed below 100 °C.

ZUKÁL (1970) — investigating the effect of long lasting heat treatment at 120 °C on purified beef myofibrils — found, that extensive changes were going on in myofibrillar protein even in the absence of other reactants (sugar, salts, *etc.*). The changes take place in the water-solubility of the protein: in other words the same proteins which become insoluble in water when heated between 60 and 100 °C, dissolve again at 120 °C. It was also suggested (SCHOBERL & HAMM, 1964), that this process might be caused by a breakdown either of hydrogen bonds or, perhaps, of disulphide bonds in the proteins.

In these experiments prolonged heating periods were choosen which are not used in meat processing. However, temperatures as high as 120 °C are commonly used in producing sterilized canned meat products. Preliminary results verified namely that the proteins of myofibrils do not suffer appreciable changes at low temperatures and/or short heating periods, concerning either nutritive value or thermal degradation (ZUKÁL, 1970). The experiments are of theoretical interest and contribute to the knowledge of the mechanism of heat decomposition of meat proteins.

1. Materials and methods

1.1. Preparation of myofibrils (purified myofibrillar protein)

Post rigor semimembranosus muscle was minced twice. One part of meat was mixed with 1.5 parts of cooled distilled water (5 °C) in a *Ultrathurrax* homogenizer (JANKE-KUNKEL T45) for 30 sec and rubbed through a sieve (1 mm mesh) to eliminate connective tissue. The gel thus obtained was centrifuged at 2 500 g for 20 min, after which one part of gel was again mixed with 1.5 parts of 1% NaCl solution (in water) and centrifuged as before. The rest was mixed with cooled water (1 : 5) and centrifuged. This washing procedure was repeated three times. The protein sediment resulting from the last centrifugation was used for the experiments. From minced pork semimembranosus muscle sarcoplasmic protein and the greater part of connective tissue has been removed. The rest, what we call purified myofibrillar protein, contained also traces of mitochondrial particles.

1.2. Heating

Samples of purified myofibrillar protein were put into cans (diametre: 7 cm, height: 3.5 cm). After sealing they were heated in a thermostat at 80 °C, or in an autoclave at 120 °C. The heating times were: 7.5, 15, 22.5, 30, 37.5 and 45 hours.

1.3. Determination of the amount of water-soluble materials

The cans were opened and the samples were suspended in boiling water. The insoluble residue was separated by filtration. This procedure with the insoluble residue was repeated until the last filtrate was free of water soluble nitrogenous compounds. The filtrates were collected. The water from the insoluble residue and from the material was evaporated (in form of an azeotropic mixture of ethanol and water) on a water bath.

1.4. Determination of non-protein nitrogen

The extraction of non-protein nitrogen was carried out with 10% trichloro acetic acid and/or 80% ethanol. 1 g of the dry material was rubbed in a mortar with 10 ml of these solutions. The nitrogen content was determined according to *Kjeldahl*.

1.5. Amino acid analysis

Amino acids were determined in 80% ethanol extract. To 2 ml of this extract (nitrogen content: 1.6 mg) 10 ml of 1% picric acid solution was added and the mixture was centrifuged (10 min, at 7 000 rpm). Picric acid was

removed from the supernatant by a column filled with Dowex 2 × 8 (STEIN & MOORE, 1954). The purified material was dissolved in 1 ml 0.2 *N* citrate buffer (pH 2.2) and the separation and determination of amino acids were made by an automatic amino acid analyser (BIOCAL).

2. Results and conclusions

Table 1 shows the composition of the pork myofibrillar protein fraction which was used throughout this experiment. The effect of heating at 120 °C on meat protein can be seen in Fig. 1. As Fig. 1 shows the water soluble fraction

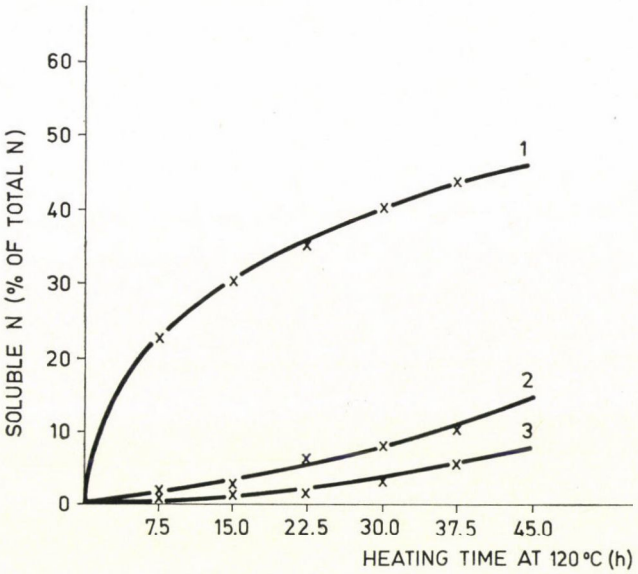

Fig. 1. Effect of heating on solubility of myofibrillar gel. 1 — in water; 2 — in 10% trichloro acetic acid; 3 — in 80% ethanol

increased steadily with heating time. The same results were obtained with beef myofibrils by ZUKÁL (1970). After 45 hours of heating 46% of the myofibrillar proteins became water-soluble. This aqueous extract contains proteins and N-compounds of low molecular weight that are soluble in trichloro acetic acid or ethanol.

The changes taking place during heating in solubility in 10% trichloroacetic acid and in 80% ethanol can be attributed to the formation of nitrogenous compounds of low molecular weight, *i.e.* to the breakdown of myofibrillar proteins. These changes were not observed at 80 °C even after heating for 45 hours.

The increase in protein N-content of the water extract during heating can be explained presumably by rearrangement of the secondary and tertiary

Table 1

Analysis of protein gel

Component	%
Dry substance (% of gel wt.)	9.09
Total nitrogen (% of dry substance)	15.18
Lipid (% of dry substance)	1.82
Connective tissue protein nitrogen (% of total nitrogen)	0.81
NaCl content (% of dry substance)	0.002

structure of the coagulated myofibrillar proteins which renders them water soluble. Further investigations should be carried out to elucidate this phenomenon.

The tests by amino acid analyser confirmed the assumption that some of the muscle proteins suffer breakdown during heating at 120 °C yielding free amino acids and peptides of low molecular weight. The changes in content of free amino acids and peptides are plotted as a function of heating time in Fig. 2. While the amount of peptides increases slowly during heating, the amount of free amino acids increases at a much higher rate, mainly from the 22.5th hour onward.

Fig. 3. shows the percentage of free amino acids, as determined with the amino acid analyser, as a function of heating time.

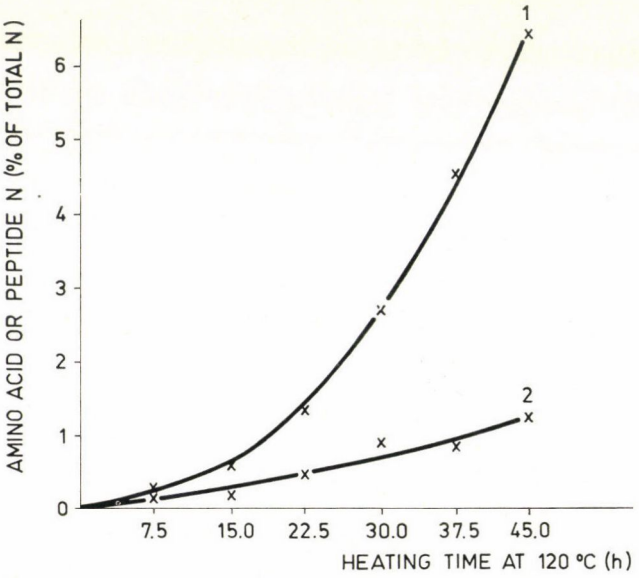

Fig. 2. Changes in content of free amino acids (1) and peptides (2) during heating

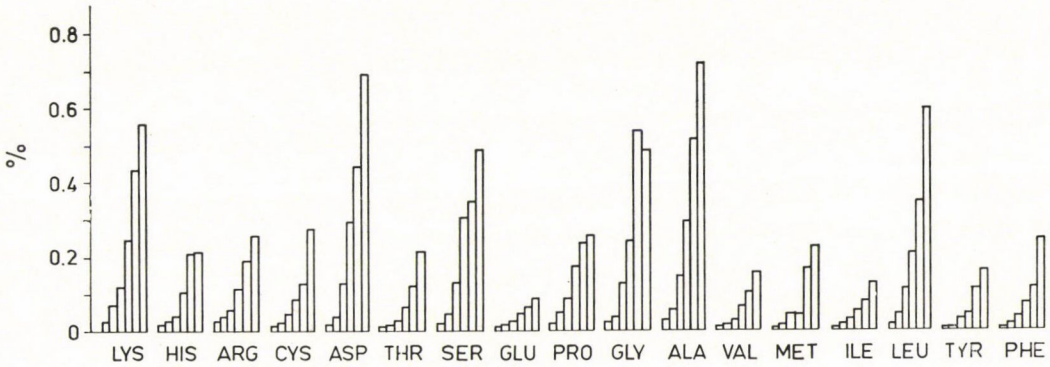

Fig. 3. Changes of free amino acids during heating (120 °C, 7.5, 15, 22.5, 30, 37.5 and 45 hours)

On the basis of our investigations it was established that free amino acids are not released from peptide linkages in the myofibrillar protein at 80 °C as mentioned earlier and they can be released only at higher temperatures, *e.g.* at 120 °C and during a long heating time.

Provided that ammonia develops below 100 °C (DROSDOV, 1938; JANITZ, 1971) this process can only be assumed if proteins are deaminated by heating, without their breakdown to peptides and/or amino acids preceding this deamination. Our results suggest that there are no peptides — and/or amino acids released from myofibrillar protein of meat during regular heating (frying excluded) generally used in practice. Heating times and temperatures generally used even in canning are namely too low and/or too short to cause such changes. Further tests are necessary in order to determine how the other protein fractions change during heating and how the meat additives (salt, polyphosphate, *etc.*) influence this phenomenon.

*

The authors wish to thank Dr. GY. JÉCSAI (RESEARCH INSTITUTE FOR ANIMAL BREEDING) for her help in amino acid analysis.

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Acta Alimentaria, Vol. 4 (4), pp. 373—380 (1975)

EFFECT OF GAMMA-IRRADIATION ON RED GRAM (*CAJANUS CAJAN*) LIPIDS

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(Received December 23, 1974)

No changes in total or neutral lipid composition are observed in red gram irradiated at 1 Mrad dose levels. Similarly, saturated and unsaturated fatty acids are not affected by radiation treatment on storage. This prevents the development of rancidity and off-flavours in irradiated samples even on storage for 8 months. The prevention of oxidation under such conditions can also be attributed to the synergistic effect of phospholipids in presence of tocopherols as well as to low moisture content. However, polar lipids seem to undergo decomposition on irradiation.

Legumes, except for oil-bearing seeds like soybean and peanut, are generally low in lipid content. Relatively little is known about the effect of radiation on changes in the lipid composition of these seeds.

The main effects of radiation on glycerides are formation of peroxides (WILLS & ROTBLAT, 1964; TIPPLES & NORRIS, 1965) and volatile carbonyl compounds (CHIPAULT, 1962; NAWAR, 1972) which are responsible for rancidity and off-flavours. Similar effects could be initiated on cooking foods rich in lipids due to autoxidation (GREENE & WALTZ, 1966). Such changes can severly affect acceptability and organoleptic rating of irradiated foods. It is known that flavour components in lipids may arise by physical transfer, heating, enzyme action or by secondary reactions (FORSS, 1967). It has been shown that unsaturated fatty acids can polymerize as well as transisomerise on irradiation at high doses (PAN *et al.*, 1957).

We have observed that gamma-irradiation of the dry legume at 1 Mrad reduces the cooking time and improves rheological as well as textural properties of red gram *(Cajanus cajan)*. These have been attributed to physicochemical changes in the main food constituents, namely, starch and proteins (NENE *et al.*, 1974a, b). The present investigation relates to the effect of gamma radiation (1 Mrad) on lipid composition of red gram. The total lipids and fatty acid profiles of unirradiated and irradiated red gram, immediately and after storage for eight months at 0 °C, were studied.

1. Materials and methods

1.1. Materials

Red gram was procured from local market. Standard fatty acid methyl esters and authentic lipid samples *viz.*, monoolein, distearin, tripalmitin, cholesterol, lecithin, cephalin, digalactosyl diglyceride, phosphatidic acid, *etc.*, were either from SIGMA CHEMICAL CO. USA, or from the UNIVERSITY DEPARTMENT OF CHEMICAL TECHNOLOGY, Bombay. The solvents were of *Analar* (BDH) quality and re-distilled before use. Silica gel was from E. MERCK, USA and Chromosorb P from APPLIED SCIENCE LAB., USA.

1.2. Irradiation

Dry red gram samples (100 g lots) were packed in polyethylene bags and exposed at 25 °C to a ^{60}Co-source (*Gamma cell* 220, ATOMIC ENERGY OF CANADA Ltd) having a flux of 15 krad min^{-1}, at 1 Mrad dose level; overdose ratio was about 30 per cent. Absorption of radiation was checked with ferrous sulphate and ceric sulphate dosimetry (WEISS, 1952).

1.3. Analytical procedures

1.3.1. Lipids. Total lipids of red gram were extracted repeatedly with chloroform and methanol (2 : 1, v/v) and last traces of carbohydrates and proteins removed from the combined extracts (FOLCH *et al.*, 1957). The purified extract was dried in vacuum, weighed to constant weight and stored under nitrogen at 0 °C. Polar lipids were isolated from total lipids by repeated precipitation with acetone. Phospholipids were estimated from total lipids by gravimetric (VACCARINO & VACCARINO, 1961) and colorimetric (FISKE & SUBBAROW, 1925) methods. Glyceride content and non-saponifiables of lipid extract were determined according to the procedure of VAN HANDEL (1961) and the method described in A. O. C. S. (1958), respectively.

1.3.2. Thin-layer chromatography (TLC). Glass plates (20 × 20 cm) were spread with silica gel slurry (prepared in distilled water, 1 : 2, w/v), dried and activated at 120 °C for 90 min. Total lipid extracts were taken in ether and a 200-μg sample spotted on the plate for separation of polar and non-polar lipids; authentic samples were run simultaneously on the same plate. The mixtures of petroleum ether (B. P. 60 °C) : diethyl ether : acetic acid (85 : 15 : : 1, v/v) and chloroform : acetic acid : water (50 : 35 : 3, v/v), were used as solvent systems to separate polar and non-polar lipids, respectively. The chromatograms were visualized by spraying the plates with saturated $K_2Cr_2O_7$ in 70% H_2SO_4, followed by charring at 150 °C for 30 min. Cephalin and lecithin were detected by spraying the developed plates with ninhydrin and *Dragendorf*'s reagent, respectively (WALDI, 1965).

1.3.3. Gas-liquid chromatography (GLC). Total lipids were saponified with alcoholic 2 *N* KOH for2 h and excess alcohol evaporated. Residue was dissolved in water and the non-saponifiables were removed (A. O. C. S., 1958). The aqueous layer was acidified and the liberated fatty acids were extracted with diethyl ether. Solvent phase was washed with water till free from mineral acid, dried over anhydrous Na_2SO_4 and the fatty acids were concentrated under a stream of nitrogen. These were then methylated by refluxing for 2 h with anhydrous methanol and 2—3 drops of conc. H_2SO_4.

Methyl esters of fatty acids thus formed were analysed in a gas chromatograph (BHABHA ATOMIC RESEARCH CENTRE Model), equipped with a flame ionization detector and a stainless steel column (6' × 0.25'') packed with 20% ethylene glycol succinate on acid-washed 60/80 mesh Chromosorb P. The carrier gas was nitrogen with a flow rate of 25 ml min^{-1}. The column and injection port were maintained at 185 °C, respectively. Fatty acid peaks were recorded on a *Shimadzu* recorder. Individual fatty acids were identified by comparing with the retention times of reference standards. Gas chromatographic peak areas were determined by multiplying peak height by peak width at half height.

2. Results

2.1. Lipid composition of irradiated red gram

Data on the effect of gamma-radiation and storage on lipid composition of red gram are presented in Table 1. Values of total lipids were comparable in control and irradiated samples even after 8 months storage. Similarly, no

Table 1

Lipid composition of unirradiated and irradiated red gram

Sample	Storage time, months	Total lipids, % dry wt.	Phospholipids		Neutral lipids		Non-saponifiables, % wt.
			Chemical	Gravimetric	Chemical[1]	Gravimetric[2]	
			% wt. of total lipids				
Control	0	2.8	27.2	30.6	75	66.8	2.6
Irradiated (1 Mrad)	0	2.8	26.6	31.9	73	65.2	2.9
Control	8	2.9	26.4	30.1	75.2	67.5	2.4
Irradiated (1 Mrad)	8	2.8	26.2	29.8	73.6	67.6	2.6

Values are averages of 3 experiments
Lipids were estimated by chemical and gravimetric methods as described in the text

[1] Represents total glycerides
[2] Calculated by difference: Total lipids – (total phospholipids + nonsaponifiables)

appreciable differences in triglycerides, the major component (about 70%) of red gram or in phospholipids (26—27%) were observed. However, estimation of phospholipid by gravimetric method resulted in relatively higher values than those by chemical method. This may be due to the presence of residual neutral lipids and possibly other trace contaminants in the acetone-precipitated phospholipid fractions.

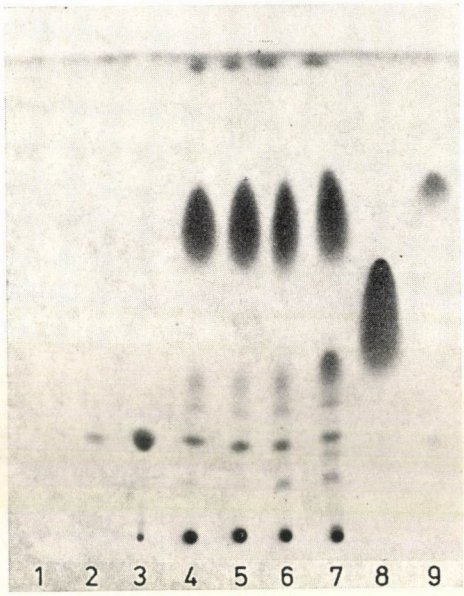

Fig. 1. Thin-layer chromatogram of non-polar lipids: This was obtained from control and irradiated (1 Mrad) red gram lipids on silica gel plate, using petroleum ether: diethyl ether : acetic acid (85 : 15 : 1) as developing solvent system. 1 — monoglyceride; 2 — diglyceride; 3 — cholesterol; 4 — control; 5 — irradiated; 6 — control (stored); 7 — irradiated (stored); 8 — free fatty acids; 9 — triglycerides

2.2. Distribution of fatty acids in lipid components of red gram

Thin-layer chromatogram (TLC) of total lipids (Fig. 1) shows the presence of mono-, di- and tri-glycerides, sterols and traces of free fatty acids amongst which the predominant one is triglyceride. The presence of phospholipids was evident from the spot at the baseline, but other minor spots were not identified. Fig. 2 shows the TLC separation of polar lipids tentatively identified as lecithin, cephalin, digalactosyl diglyceride, phosphatidic acid and some unidentified minor spots. The polar lipids seem to be decomposed on irradiation since the spots are less intense and cover lesser area. These changes were not discernible when chemical or gravimetric method was employed. A typical gas chromatogram of fatty acids of red gram is shown in Fig. 3. Results on the distribution of fatty acids in total, neutral and phospho-

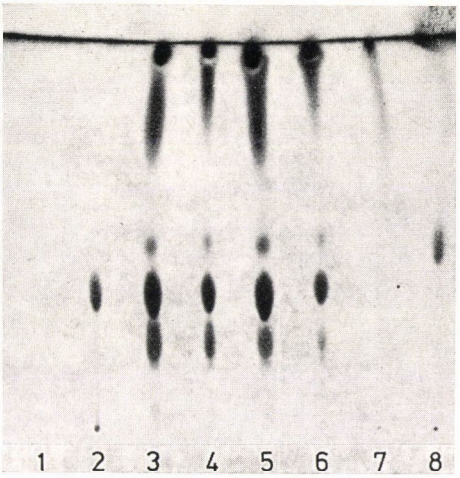

Fig. 2. Thin-layer chromatogram of isolated polar lipids: Polar lipids, isolated from control and irradiated (1 Mrad) red gram samples, were separated on silica gel plate using chloroform : acetic acid : water (50 : 35 : 3) as developing solvent system. 1 — cephalin; 2 — lecithin; 3 — control; 4 — irradiated; 5 — control (stored); 6 — irradiated (stored); 7 — phosphatidic acid; 8 — digalactosyl diglyceride

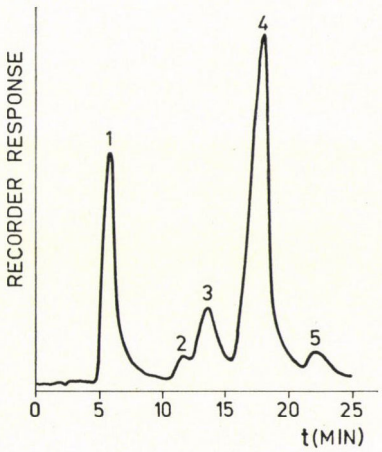

Fig. 3. Gas chromatogram of fatty acids of red gram. The chromatogram was obtained by the procedure described in "Materials and Methods". The peaks represent: 1 — palmitic acid; 2 — stearic acid; 3 — oleic acid; 4 — linoleic acid; 5 — linolenic acid, on the basis of standard samples

lipids are summarized in Table 2. Total saturated fatty acids (16 : 0, 18 : 0) were relatively more in phospholipids fraction (35%) than in the neutral lipids (25.6%). Linoleic acid contributed about 50% of the total unsaturated fatty acids in the phospholipid and glyceride fractions. Oleic and linolenic acids were found to be relatively more in the neutral lipids (14.4 and 7.7%,

Table 2

Distribution of fatty acids (% wt.) in lipid components of red gram

Fatty acids	Total lipids		Phospholipids		Neutral lipids		Total lipids (stored samples)	
	Control	Irr.	Control	Irr.	Control	Irr.	Control	Irr.
Palmitic	22.7	24.7	30.5	32.7	20.4	22.8	22.8	24.9
Stearic	5.8	5.8	4.5	4.4	5.2	5.0	4.0	4.0
Oleic	14.1	13.8	8.9	8.5	14.4	15.5	12.4	10.4
Linoleic	51.3	49.0	52.6	51.7	52.2	50.0	55.7	54.9
Linolenic	6.0	6.6	3.1	2.6	7.7	6.7	5.4	5.7

Results are averages of three experiments. Distribution of fatty acids separated by TLC (Fig. 3), in various lipid fractions was carried out in control, irradiated (1 Mrad) and stored (for 8 months) samples.

respectively) than in the phospholipid (8.9 and 3.1%, respectively) fractions. No appreciable changes were observed in the fatty acid composition of the lipid fractions of red gram irradiated at 1 Mrad.

3. Conclusions

Earlier, we had investigated the potentiality of radiation processing for improving the cooking quality of dry legumes. It was observed that longer cooking time, which generally limits the use of these otherwise cheap and protein-rich foods, was reduced significantly in irradiated samples, particularly in red gram (NENE *et al.*, 1974c). It is known that the lipid component of food influences its nutritional value, stability on storage and other processing characteristics. Red gram belongs to the category of legumes low in oil content (WOLFF & KWOLEK, 1971) since its lipid content varies between 2—3% (Table 1). However, it is rich in phospholipids (26% of total lipids), which are known to influence the textural qualities of foods (NARAYANAN & HLYNKA, 1962; KATSUHARU *et al.*, 1964; POMERANZ *et al.*, 1970). It was observed that radiation treatment improves the texture of red gram (NENE *et al.*, 1974c).

The principal effect of radiation on lipids involves direct cleavage of C—C bonds, especially in the unsaturated fatty acids (CASARETT, 1968). In the presence of oxygen, organic peroxides and hydroperoxides are formed similar to autoxidation process leading to aldehyde and ketone formation. These reactions are of special importance since about 70% of fatty acids in red gram are unsaturated (Table 2). However, unsaturated or saturated fatty acid composition of red gram, irradiated at 1 Mrad, was not changed appreciably, even after storage for eight months. This may be attributed

to the presence of natural antioxidants like tocopherols (DATE, 1955); red gram contains about 46 mg tocopherol per 100 g lipids (NAZIR & MAGAR, 1963) and this may be responsible for the stability of red gram flour up to 4 months, the samples being free from rancidity even at 37 °C. Moreover, red gram contains appreciable amounts of lecithin (Fig. 2) which may also contribute to the stability of lipids (neutral) in irradiated samples. Phospholipids, in general and lecithin in particular are known to synergize the action of antioxidants probably by donating hydrogen or an electron (STUCKEY, 1962).

In complex foods of low moisture content, water itself can give quite a powerful protection against lipid oxidation (LEA, 1962). It has been shown that, for dehydrated foods, storage at moisture levels above monolayer coverage, gives maximum resistance to oxidation (SALWIN, 1959). The moisture content of red gram was found to be about 12%. The probable mechanism of the protective effect of water against lipid oxidation has been investigated (MALONEY *et al.*, 1966; LABUZA *et al.*, 1966; KAREL *et al.*, 1967). Lower water content in red gram may thus also contribute to protection of lipids against radiation induced oxidation.

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Acta Alimentaria, Vol. 4 (4), pp. 381—390 (1975)

POLYPHENOLIC COMPOUNDS IN TISSUES OF BRINJALS *(SOLANUM MELONGENA)*

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(Received January 7, 1975)

The polyphenolic compounds of brinjal tissue *(Solanum melongena)* were studied by paper chromatography, spectrophotometry and colour reactions. Besides caffeic acid derivatives, p-coumaric and cinnamic acid derivatives of varying degrees of glycosylation were found to be common among the eleven compounds isolated of which four were glycosides. The sugar moieties of the glycosides were arabinose or glucose.

During preparation, processing and storage fruits and vegetables are susceptible to spoilage by discolouration. Apart from the well-known sugar–amine reactions, phenolic compounds that are widely distributed in the plant kingdom, are known to contribute extensively to the discolourations, both through enzymic and non-enzymic reactions. Brinjal fruit *(Solanum melongena)* is one vegetable that is highly susceptible to both these types of discolourations. The occurrence of chlorogenic, neochlorogenic and caffeic acids which give brown colour with purified polyphenoloxidase in egg-plant has been reported (SAKAMURA & OBATA, 1963). An active principle from the alkaline hydrolysate of purified extracts of *Solanum melongena* was identified as caffeic acid (BENIGNI *et al.*, 1958).

In the present study, an attempt was made to characterize the phenolic compounds present in brinjal fruit.

1. Materials and methods

A purple variety of brinjal *(Solanum melongena)* of average size, 6 cm long and 5 cm in diameter, was employed in this study. The authentic phenolic compounds were obtained from SIGMA CHEMICALS COMPANY, USA, and sugars from NUTRITIONAL BIOCHEMICAL CORPORATION, Cleveland, Ohio.

1.1. Extraction of polyphenols

The method employed was essentially a modification of that used by RIVAS and LUH (1968) for the extraction of polyphenolic compounds in canned tomato pastes. The chilled brinjals (1 kg), after peeling so as to avoid interference by the skin pigments, were cut into pieces and blended for 5 min with

1 litre of chilled distilled methanol. The homogenate was filtered through a muslin cloth and then through Whatman No. 1 filter paper on a *Buchner* funnel. The residue was extracted twice more with the same amount of solvent and filtered as before. The filtrates were combined and concentrated in a flash evaporator. The residue was suspended in distilled water (25 ml) and extracted several times with 10 ml portions of petroleum ether to remove the carotenoids. The water extract was saturated with sodium chloride and then extracted five times with 250 ml portions of ethyl acetate. The ethyl acetate extracts were combined, dried with anhydrous sodium sulphate, filtered and evaporated to a small volume in a flash evaporator. The residue was dissolved in 15 ml of methyl alcohol and preserved in a well-stoppered amber coloured bottle in a refrigerator.

1.2. Two-dimensional paper chromatography

The chromatographic procedure employed was a modification of the method used by WILLIAMS and WENDER (1952). A 100-μl aliquot of the extract was used for two-dimensional ascending chromatography on Whatman No. 1 paper (35 cm $\times$ 35 cm) at 25 °C with 2% acetic acid as first solvent for 3 hrs and n-butanol – acetic acid – water [BAW, 4 : 1 : 5 (v/v)] as second solvent for 15 hrs. The air-dried chromatograms were examined under ultra violet light and on exposure to ammonia vapour (SWAIN, 1953). One chromatogram was sprayed with freshly prepared $FeCl_3$—$K_3Fe(CN)_6$ reagent (KEPPLER, 1957), rinsed with 2% HCl and finally with distilled water. The blue spots, of polyphenolic compounds were detected and R_f values measured.

Four additional chromatograms were sprayed separately with diazotized p-nitroaniline (DPNA) (SWAIN, 1953), *Hoepfner* reagent (WALKER, 1962), vanillin reagent (SWAIN & HILLIS, 1959) and sodium borohydride (HOROWITZ, 1957). The colour characteristics of the spots were noted.

1.3. Acid hydrolysis of phenolic compounds

The individual spots from unsprayed chromatograms were eluted with 5 ml absolute methanol and hydrolysed in 5 ml of 10% aqueous HCl in a sealed tube kept in a boiling water-bath for 45 min (HARBORNE, 1965). The aglycones were extracted with ethyl acetate, concentrated under vacuum and chromatographed in two dimensions using 2% acetic acid and BAW (4 : 1 : 5) as developing solvents. Aglycones were detected from the blue colour with $FeCl_3$—$K_3Fe(CN)_6$ reagent (KEPPLER, 1957).

The aqueous fraction was co-chromatographed ascendingly with authentic sugars using BAW (4 : 1 : 5). This chromatogram was sprayed with aniline hydrogen-phthalate reagent (WILSON, 1959), air-dried and heated in an oven to 105 °C for 2 min for detection of sugar spots.

1.4. Absorption spectra

UV and visible absorption spectra of the chromatographically purified compounds were recorded in a *Beckman DK-2 Recording Spectrophotometer*. A 5% ethanolic $AlCl_3$ solution was used to study the shift in absorption peak due to chelation of phenolic compounds containing ortho-dihydroxy configurations.

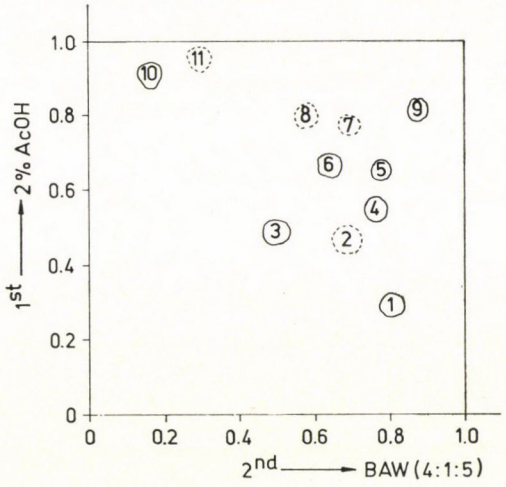

Fig. 1. Two-dimensional paper chromatogram of polyphenolic compounds in brinjal (Glycosides are marked with broken lines)

1.5. Estimation of relative amounts of polyphenolics

The phenolic compounds at different spots from the chromatograms were eluted with ethyl acetate and concentrated. After re-dissolving in water the relative amounts of phenolics were determined by the *Folin–Denis* colorimetric method described by SWAIN and HILLIS (1959) using tannic acid as standard.

2. Results

2.1. Chromatographic behaviour

A chromatogram of the methanol extract, sprayed with $FeCl_3$—$K_3Fe(CN)_6$ reagent is shown diagrammatically in Fig. 1. As this extract contained the complete spectrum of phenolics including their glycosides, it served as a reference for comparing with a chromatogram which was obtained after hydrolysis of the extract.

The number of spots detectable with UV and $FeCl_3$—$K_3Fe(CN)_6$ reagent on the chromatogram as indicated in Fig. 1 were eleven. The spots which disappeared after acid hydrolysis were Nos. 2, 7, 8 and 11 and are marked with broken lines in Fig. 1. These were considered to be the phenolic glycosides.

Table 1 shows the R_f values and colour reactions of the phenolic compounds in methanol extract of brinjal and authentic polyphenolics in the two solvent systems — 2% acetic acid and BAW (4 : 1 : 5).

The common substrates for enzymatic oxidation by polyphenoloxidase are chlorogenic and caffeic acids (Rivas & Luh, 1968). When 2% acetic acid and BAW (4 : 1 : 5) were used as solvent (Fig. 1), it was observed that none of the spots of polyphenolic compounds isolated from brinjal tissues corresponded to those of caffeic, ferulic and chlorogenic acids as given in Table 1 (Hanson & Zucker, 1963; Van Bragt *et al.*, 1965; Zane *et al.*, 1965). Spots 3, 4, 5, 7, 8 and 11 seem to belong to the category of phenolic acids as they answer DPNA test on the chromatogram. Their R_f values however, did not

Table 1

R_f values and colour reactions of phenolic compounds present in brinjal

Spots	R_f values		UV	UV + NH_3	Visible	Visible + NH_3	$FeCl_3$—$K_3Fe(CN)_6$	Diazotized p-nitro aniline	*Hoepfner* reagent	Sodium borohydride	Vanillin-HCl
	BAW (4:1:5)	2% AcOH									
1	0.81	0.30	P	P	—	F Y	Bl	—	—	—	—
2	0.68	0.48	D P	P	—	F Y	Bl	—	—	—	—
3	0.50	0.48	Bl	Y G	—	Y	Bl	R Br	Y	—	—
4	0.77	0.55	P	Y G	—	Y	Bl	Y Br	Y	—	—
5	0.77	0.65	P	Y G	—	Y	Bl	Y	Y	—	—
6	0.63	0.66	V	D P	—	Y	Bl	—	—	—	—
7	0.69	0.78	F P	F Y	—	F Y	Bl	Y Br	Y	—	—
8	0.57	0.80	F P	F Y	—	F Y	Bl	Y	Y	—	—
9	0.86	0.81	Br Y	Y	—	Y	Bl	—	—	—	—
10	0.16	0.90	Br	Br Y	Br	Y Br	Bl	—	—	—	—
11	0.28	0.95	Br Y	Br Y	—	Y Br	Bl	L Y	L Y	—	—
Authentics											
Ferulic acid	0.81	0.45	Bl	Bl	C	C	Bl	R	Y Br	C	—
Caffeic acid	0.71	0.23	Bl	Bl	C	C	Bl	Br	R	C	—
Chlorogenic acid	0.61	0.70	Bl	Y G	C	Y	Bl	T	Y	C	—

Bl = Blue, Br = Brown, C = Colourless, D = Deep, F = Faint, G = Green, L = Light, P = Purple, R = Red, T = Tan, V = Violet, Y = Yellow, — indicates negative test

agree with those of the known phenolic acids: caffeic, ferulic and chlorogenic. The possibility that these compounds may be certain derivatives of these acids may not be precluded. Several such derivatives like neochlorogenic, isochlorogenic and dactylifric acids have been reported (CORSE, 1964). The negative test shown for both vanillin reagent and sodium borohydride reagent revealed that flavonols or their derivatives were absent among the eleven compounds isolated. Spot 10 appeared blackish brown, when the chromatogram was dried. This may probably be a product of non-enzymic browning reaction. The intensity of this spot increased with the concomitant decrease in other phenolics on the chromatogram, when the fresh vegetable extract was allowed to undergo oxidation before the phenolic compounds were extracted and chromatographed.

Table 2 and 3 represent the R_f and R_G values of the aglycones and sugar moieties of the glycosides, respectively. R_f values of aglycones as shown in Table 2 were comparable to spots 1, 3, 4, 5, 6, 9 and 10 of the chromatogram developed with unhydrolysed extract (Fig. 1). The missing spots were expected to be of glycosides of polyphenols, the sugar moieties of which were glucose and arabinose (Table 3). When glycosides extracted from spots 2, 7, 8 and 11 were individually hydrolysed and subjected to chromatography with the two solvent systems [2% acetic acid and BAW (4 : 1 : 5)], their aglycones correspond with spots 1, 5, 3 and 4, respectively. Whereas spots 2 and 11 contained arabinose, glucose was detected in spots 7 and 8. Considering the nature of separation of spots 3 and 8 on the paper chromatogram (Fig. 1), the glycoside nature of spot 8 was revealed from its higher R_f value in 2% acetic acid system. Glycosides always run slower in t-butanol–acetic acid–water (3 : 1 : 1) and faster in 15% acetic acid systems than do their corresponding aglycones as observed with crude plant extract of *Baptisia lecontei* (MARKHAM & MABRY, 1968). Such a relationship was also noted in the aglycone–glycoside pairs denoted by spots Nos. 1 and 2, 4 and 11 and 5 and 7. The higher mobility of spot 8 in the present work in BAW (4 : 1 : 5) system than spot 3, in spite of the former probably being a glycoside of the latter, may only be explained on the assumption of its higher methoxy content. It is reported that methyl ethers of polyphenols show a faster movement in t-butanol–acetic acid–water (3 : 1 : 1) system than in 15% acetic acid system (MABRY *et al.*, 1970).

2.2. Spectral characteristics

It is observed from Table 4 that compounds 1, 2, 4, 5, 6, 9, 10 and 11 have almost similar UV absorption spectra with and without $AlCl_3$. Perhaps all these compounds are closely related having the same ring structure with different substitutions. Only compounds 3 and 8 showed distinct characteristics.

Table 2

R_f values of aglycones of phenolic glycosides

Aglycones		R_f value	
		BAW (4 : 1 : 5)	2% acetic acid
Spots	1	0.81	0.30
	3	0.50	0.48
	4	0.77	0.55
	5	0.77	0.65
	6	0.63	0.66
	9	0.86	0.81
	10	0.16	0.90

Table 3

R_G values of sugar moiety present in glycosides

Samples	R_G value in BAW (4 : 1 : 5) system
Acid hydrolysed portions of spots	
2	118, 117
7	100
8	100
11	118, 117
Standards	
Glucose	100
Galactose	89
Arabinose	117
Rhamnose	206

Of these compounds 3 exhibited pattern similar to caffeic acid derivative as reported by RIVAS and LUH (1968). Like methylation and acetylation, glycosylation has a hypsochromic effect on spectra of various polyhydroxy systems, particularly polyphenols (HARBORNE, 1964a). Similar effect was observed in case of the spots 2, 7, 8 and 11, when their λ_{max} were compared with those of the corresponding aglycones, spots 1, 5, 3 and 4, respectively. It was also observed that the λ_{max} of aglycones of compounds 2, 7, 8 and 11 corresponded to those of 1, 5, 3 and 4. The possibility that these compounds may be hydroxy aldehydes, ketones or acid derivatives is ruled out as they have only a principal

Table 4

Spectral characteristics of phenolics isolated from brinjal

Compounds		λ_{max} (nm)		λ_{min} (nm)		λ_{max} with $AlCl_3$ (nm)	
Spot	1	253	277	243	—	256	279
	2	252	275	244	—	256	275
	3	323	294	264	—	327	299
	4	255	278	244	—	256	282
	5	250	285	241	—	252	286
	6	253	282	243	—	258	289
	7	239	281	241	—	246	288
	8	307	278	263	—	313	286
	9	255	283	250	273	259	288
	10	252	284	244	275	258	286
	11	249	271	244	—	257	279
Standards							
Ferulic acid		320	—	—	—	—	—
Caffeic acid		328	298	262	—	—	—
Chlorogenic acid		325	295	265	—	328	300

absorption maximum within a range of 249—295 nm while the latter ones have both principal and subsidiary maxima at 255—290 nm and at 300—350 nm, respectively (MORTON & STUBBS, 1940; LEMON, 1947; CRAM & CRANZ, 1950). On the basis of absorption spectra and in particular the λ_{max}, all the compounds appeared to correspond to the class of cinnamic acid and/or coumarin. Their shifts in λ_{max} in $AlCl_3$, however, do not tally with the value of 20 nm as reported by HARBORNE (1964b) showing that all the compounds perhaps have a high degree of methylation. The tentative identification of the individual compounds on the basis of the above observations is given in Table 5.

2.3. Relative amounts of polyphenolics

When the quantity of each phenol was estimated by the *Folin–Denis* colorimetric method (SWAIN & HILLIS, 1959) using tannic acid as standard, the four major spots 2, 4, 6 and 11 were found to be present in amounts equivalent to 9.8, 10.2, 9.7 and 12 μmoles of tannic acid, respectively (Table 6). The total phenolics present in the ethyl acetate extract of 1 kg brinjal tissue were 85.7 μmoles. In spite of this small concentration, the phenolics introduce a lot of difficulties in the processing of the vegetable.

Table 5

Tentative identification of phenolic compounds in brinjal

Spot	R_f values		On hydrolysis		Type of functional group		Spectral characteristics		Tentative identification
	BAW (4 : 1 : 5)	2% AcOH	Sugar	Aglycone	$FeCl_3$—$K_3Fe(CN)_6$ reagent	DPNA and *Hoepfner* reagent	λ_{max} (nm)	λ_{max} (nm) in $AlCl_3$	
1	0.81	0.30	Nil	Agly. (2)	Cat./pyr.	Nil	253, 277	256, 279	Coumaric or cinnamic acid derivative
2	0.68	0.48	Arab.	Gly. (1)	Cat./pyr.	Nil	252, 275	256, 275	Arabinoside of No. 1
3	0.50	0.48	Nil	Agly. (8)	Cat./pyr.	Phenolic acid	323, 294	327, 299	Caffeic acid derivative
4	0.77	0.55	Nil	Agly. (11)	Cat./pyr.	Phenolic acid	255, 278	256, 282	Coumaric or cinnamic acid derivative
5	0.77	0.65	Nil	Agly. (7)	Cat./pyr.	Phenolic acid	250, 285	252, 286	Coumaric or cinnamic acid derivative
6	0.63	0.66	Nil	Unknown	Cat./pyr.	Nil	253, 282	258, 289	Coumaric or cinnamic acid derivative (?)
7	0.69	0.78	Glu.	Gly. (5)	Cat./pyr.	Phenolic acid	239, 281	246, 288	Glucoside of No. 5
8	0.57	0.80	Glu.	Gly. (3)	Cat./pyr.	Phenolic acid	307, 278	313, 286	Glucoside of No. 3 with methoxyl grouping
9	0.86	0.81	Nil	Unknown	Cat./pyr.	Nil	255, 283	259, 288	Coumaric or cinnamic acid derivative (?)
10	0.16	0.90	Nil	Non-enzymic oxidation brown products	Cat./pyr.	Nil	252, 284	250, 286	Coumaric or cinnamic acid derivative (?)
11	0.28	0.95	Arab.	Gly. (4)	Cat./pyr.	Phenolic acid	249, 271	257, 279	Arabinoside of No. 4

Agly. = Aglycone, Gly. = Glycoside, Arab. = Arabinose, Glu. = Glucose, Cat. = Catechol, pyr. = Pyrogallol

Table 6

Relative amounts of polyphenolic compounds present in ethyl acetate extract

Compounds		Total phenolic content (Tannic acid equivalent, μmoles	Relative amounts, %
Spot	1	5.525	6.42
	2	9.775	11.14
	3	6.375	7.50
	4	10.200	11.90
	5	3.910	4.59
	6	9.690	11.30
	7	6.555	7.70
	8	6.880	7.95
	9	8.500	9.90
	10	6.460	7.60
	11	11.950	14.00

3. Conclusions

The polyphenolic compounds present in tissues of brinjals *(Solanum melongena)* were extracted with methanol and ethyl acetate. The compounds were separated by two dimensional paper chromatography with 3% acetic acid and n-butanol–acetic acid–water (4 : 1 : 5) systems. The identification of individual compounds was tried on the basis of their R_f values and colour reactions with $FeCl_3$—$K_3Fe(CN)_6$, diazotized p-nitroaniline, *Hoepfner*, sodium borohydride and vanillin-HCl reagents, fluorescent behaviour and absorption spectra. Of the eleven phenolic compounds detected on the chromatogram, only four were found to be glycosides. The sugar moieties of these were found to be arabinose or glucose. These phenolics seem to belong to the class of cinnamic, coumaric or caffeic acid derivatives. The relative amount of these phenolics was determined on the basis of the colour reaction.

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Acta Alimentaria, Vol. 4 (4), pp. 391—404 (1975)

PRODUCTION OF CHEESE WITH A MILK CLOTTING ENZYME PREPARATION OF MICROBIAL ORIGIN

PART II. — TOTAL AND SOLUBLE PROTEIN CONTENT OF CHEESES

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(Received February 26, 1975)

Investigations have been carried out with batches of Emmental, Trappist, "Pálpusztai" and White cheese manufactured in parallel with calf stomach rennet and a microbial milk clotting enzyme preparation developed in this laboratory by submerged fermentation of an *Endothia parasitica* strain. The aim of the study was to establish the differences in chemical and sensory characteristics as might be induced by the use of the latter coagulant. The present paper deals with the comparison of total protein content as well as of the relative amounts and the composition of water and alcohol soluble protein degradation products.

The type of the coagulant did not seem to affect the total protein content of hard and semi-hard cheese (Emmental and Trappist, resp.) as manufactured from cow's milk. The microbial enzyme preparation had a marked, but opposite effect on the total protein content of the soft cheeses investigated: in the majority of cases it caused an increase in the total protein content of "Pálpusztai" manufactured from cow's milk and a decrease in the respective characteristic of White cheese manufactured from ewe's milk.

The changes in soluble protein degradation products as induced by the use of the microbial enzyme preparation were slight in Emmental. The results obtained with Trappist cheese were not unambiguous and are, therefore, difficult to interpret. It might be stated with some certainty, that the relative concentration of higher molecular weight (alcohol insoluble) protein breakdown products was decreased by the microbial rennet. The most striking differences in the soluble protein degradation products as induced by the rennet used were observed in soft cheese. In both "Pálpusztai" and White cheese the content of alcohol soluble protein degradation products was considerably increased by the use of the microbial preparation. Hence, this coagulant seems to promote the formation of low molecular weight nitrogenous compounds (free amino acids and small peptides). The composition of solubilized protein in soft cheese as established by gel electrophoresis was found to depend on the type of rennet used in the case of "Pálpusztai" but not in that of White cheese.

No relationship could be established between protein content and/or composition of cheese and variations in the amount of microbial rennet as applied in the manufacturing process.

In a previous paper (VÁMOS-VIGYÁZÓ *et al.*, 1975b) an account was given on the amount and composition of the free fatty acids and those present as salts in various cheese types manufactured in Hungary as well as on the changes of these characteristics as induced by the use of a microbial rennetting agent instead of calf stomach rennet. The present study is concerned with another important group of cheese constituents, *i.e.* the soluble protein degradation products (PDP) formed during ripening and with the differences in their respective amounts and compositions as ob-

served when comparing cheese batches manufactured in parallel with calf rennet and the microbial milk clotting enzyme preparation developed in this laboratory by submerged fermentation of an *Endothia parasitica* strain.

1. Materials and methods

1.1. Enzyme preparations

The calf stomach rennet used was a commercial product standardized with NaCl to an enzyme concentration of 50 000 Soxhlet Units (SU) per gram and manufactured by the SUPPLY ENTERPRISE OF ANIMAL MARKETING AND MEAT INDUSTRY, Budapest.

The microbial rennet was a powdered experimental product of 75 000 SU g^{-1} enzyme concentration containing no additives (KISS *et al.*, 1973).

Concentration, in Su g^{-1}, is measured by the amount of milk, in ml, coagulated by 1 g of enzyme preparation in 40 min at 35 °C (BALATONI, 1960).

1.2. Cheese types

The cheese types investigated were the same as dealt with in Part I of this series, with the exception of "Tea"-cheese which has not been included in the present study. The batches of Emmental, Trappist, "Pálpusztai" and White cheese treated here are only partly identical with those investigated for fatty acid composition. The signs of the batches, the year of production and the type and amount of the rennet used are given in Tables 1—4 along with some of the results.

Batches of a given type of cheese prepared in parallel with calf rennet *(CR)* and microbial rennet *(MR)* bear identical first or first and second numerals and have been manufactured — apart from rennet concentration — under strictly identical conditions. Ripening, too, occurred under identical conditions and investigations were carried out at the mature state as described in the preceding paper.

CR was added in standard amounts, while the concentrations of *MR* were varied to establish the value yielding cheese of optimum sensory characteristics. Thus in some cases different concentrations (clotting powers) of the two preparations were applied in parallel cheese making experiments. This problem will be dealt with in detail in the 4th paper of this series.

1.3. Determination of total protein and of soluble protein degradation products (PDP)

Total protein as well as water and alcohol soluble PDP were determined by the *Kjeldahl*-method.

The extracts of soluble PDP were prepared as follows: 4 g of cheese grated after removing the rind were ground in a mortar with 40—50 °C distilled water, transferred to a volumetric flask of 100 ml, diluted with water near to the mark, shaken and allowed to stand overnight at 5 °C. Then the volume was made up to the mark and the suspension filtered. 2 ml of the filtrate were used to determine nitrogen content.

An aliquot of the filtrate was diluted with 4 volumes of 96% alcohol and filtered. This filtrate was then concentrated to the original volume by evaporation on a water bath. 2 ml of this solution were used to establish the content of alcohol soluble PDP.

Total protein was related to cheese weight, water and alcohol soluble PDP were expressed as protein (N · 6.25) and related to total protein.

1.4. Gel electrophoretic separation of water soluble PDP

The water soluble PDP of soft cheeses were studied by polyacrylamide gel electrophoresis, too.

Gel electrophoresis was carried out according to DAVIS (1964). Water soluble PDP were extracted from cheese as described in para. 1.2. No desalting was found necessary prior to electrophoresis according to earlier findings (VÁMOS *et al.*, 1973). A dilution series was prepared from the filtered solution of PDP by adding various amounts of a 40% sucrose solution. 0.1 ml portions of these dilutions were applied on top of the gel tubes to establish, in a preliminary run, the concentration yielding the greatest number of fractions on electrophoresis. Optimum dilution proved different for different cheese types and was found to vary between 0.03 and 0.22 mg protein per gel. Volumes and dilutions applied per gel tube were kept identical within one type of cheese, irrespective of the values of soluble PDP of the various batches.

Electrophoresis was performed in an "*Analytical Acrylophor*" (PLEUGER, Belgium) applying 4 mA per gel tube. In order to achieve better resolution the apparatus was operated in a +5 °C refrigerator. By the end of the runs (after 35 to 40 min) temperature in the apparatus rose to about 15 °C.

The protein zones were stained with Amido Black 10 B and mobilities related to bromophenol blue were calculated. Under the given conditions bromophenol blue migrated 3.5 to 4.3 cm in the gel tubes of 6 cm length.

Attempts were made to quantitate electrophoretic data with a *Chromoscan* densitometer (JOYCE–LOEBL, UK). Optical densities of the stained protein zones were read at 620 nm. Peak areas were automatically and continuously integrated by the instrument, the values corresponding to individual peak areas had to be read by the operator. This caused difficulties with overlapping areas which could only be evaluated as a single peak. Overlapping of peaks occurred even with electrophoretically well separated, distinct bands

if they were located close to each other. This is due to the fact that the rate of paper displacement cannot be varied in this instrument. 4 gels each from 2 separate electrophoretic runs were evaluated in parallel for each of the cheese batches investigated by electrophoresis.

Considering the different dye binding capacities of the individual protein fractions, interpretation of these results will be restricted to cheese batches of the same type.

1.5. Mathematical treatment of the results

The mean values and standard deviations of parallel determinations were calculated and results obtained with cheese batches manufactured in parallel with the two kinds of rennet were compared by *Student*'s *t* test (SVÁB, 1973).

2. Results

2.1. Total protein content and soluble PDP of different cheese types

2.1.1. Emmental cheese. The values of total protein and soluble PDP content of Emmental cheese batches manufactured in parallel with *CR* and *MR* are summarized in Table 1.

Table 1

Comparison of total and soluble protein content of Emmental batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch	Year of production	Type of rennet used	Concentration of rennet added to 100 l milk, SU · 10^3	Protein content of cheese					
				total % (w/w)		water soluble		alcohol soluble	
						related to total protein, %			
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
1	1972	*CR*	210	25.4	0.0	44.9	0.2	19.9	0.5
1.1	1972	*MR*	210	24.9ø	0.3	38.7***	0.8	16.2***	0.4
2	1973	*CR*	130	26.9	0.1	41.7	0.5	24.5	0.1
2.1	1973	*MR*	160	26.4ø	0.1	42.2ø	0.5	21.4***	0.1
2.2	1973	*MR*	130	26.5ø	0.1	42.6ø	0.3	20.9***	0.2

$\bar{x}$ = mean of 3 parallel determinations
s = standard deviation
ø = difference not significant
*** = difference significant at the probability level of 99.9% as compared to the batches manufactured with *CR*

Batches marked with identical first numerals have been produced in parallel with *CR* and *MR*, resp., from the same milk batch.

Total and soluble protein, resp., were determined by the *Kjeldahl*-method and expressed as N · 6.25 (see para. 1.2).

As can be seen, the type of rennet had no influence on the value of total protein. In cheese manufactured with *MR* the relative concentration of alcohol soluble PDP was lower. The difference was particularly marked in the batches rennetted with a higher enzyme concentration (batches 1 and 1.1).

2.1.2. Trappist cheese. Trappist cheese has been extensively studied for several years from the point of view of protein content and protein degradation during ripening. Unfortunately, data as to the alcohol soluble PDP are available only for a small part of the batches. The results are presented in Table 2.

Table 2

Comparison of total and soluble protein content of Trappist batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch	Year of production	Type of rennet used	Concentration of rennet added to 100 l milk, SU · 10^3	Protein content of cheese					
				total % (w/w)		water soluble		alcohol soluble	
						related to total protein, %			
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
3	1971	*CR*	250	29.4	0.2	22.5	0.7	—	—
3.1	1971	*MR*	250	29.8^{ø}	0.3	23.0^{ø}	0.6	—	—
4	1971	*CR*	250	29.7	0.2	27.7	0.5	—	—
4.1	1971	*MR*	250	29.3^{ø}	0.2	32.0*	0.4	—	—
4.2	1971	*MR*	250	29.4^{ø}	0.2	28.6*	0.6	—	—
5	1971	*CR*	250	29.7	0.4	28.7	0.3	—	—
5.1	1971	*MR*	250	29.6^{ø}	0.0	26.3*	0.0	—	—
6	1973	*CR*	250	28.1	0.1	31.6	0.4	9.2	0.3
6.1	1973	*MR*	200	27.3^{ø}	0.2	30.6*	0.5	14.3***	0.6
6.2	1973	*MR*	250	27.0^{ø}	0.1	25.5***	0.7	13.4***	0.2

* = difference significant at the probability level of 95% as compared to the batches manufactured with *CR*

— = not determined

For the rest of symbols and for explanations see Table 1.

As in the case of Emmental, no significant differences in total protein content could be observed between either of the batches manufactured in parallel with different types of rennet. The relative concentration of water soluble PDP seems, on the contrary, to be affected by the coagulant. With the exception of one pair of batches (3 and 3.1) significant differences were found between cheese produced in parallel with *CR* and *MR*, resp. In 4 out of 5 batches (4.2, 5.1, 6.1 and 6.2) the use of *MR* decreased the water soluble PDP content as compared to the values obtained for cheese prepared with *CR*, while the comparison of the batches 4 and 4.1 showed an opposite effect.

Alcohol soluble PDP content was increased by the use of *MR*, contrary to the results obtained with Emmental. The slight variation in *MR* concentration as applied with batch 6.1 did not alter the trends of the results.

2.1.3. "Pálpusztai" cheese. The results obtained with various batches of this soft cheese manufactured from cow's milk are summarized in Table 3.

Table 3

Comparison of total and soluble protein content of "Pálpusztai" cheese batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch	Year of production	Type of rennet used	Concentration of rennet added to 100 l milk, SU · 10^3	Protein content of cheese					
				total % (w/w)		water soluble		alcohol soluble	
						related to total protein, %			
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
7	1973	*CR*	250	22.2	0.2	81.7	0.6	13.3	0.9
7.1	1973	*MR*	250	23.1*	0.3	59.2***	0.8	20.6***	0.5
8	1973	*CR*	220	21.9	0.1	96.5	0.6	16.9	1.6
8.1	1973	*MR*	175	23.2**	0.3	90.6***	0.1	25.1***	0.4
9	1974	*CR*	160	20.7	0.1	84.3	0.2	15.3	0.6
9.1	1974	*MR*	160	22.0***	0.1	92.2**	1.0	30.3***	0.9
10	1974	*CR*	160	21.4	0.0	84.6	0.9	21.0	0.4
10.1	1974	*MR*	160	21.0*	0.2	97.9***	0.6	30.9***	0.8

** = difference significant at the probability level of 99% as compared to the batches manufactured with *CR*

For the rest of symbols and explanations see Tables 1 and 2.

Data obtained both for total protein content and for soluble PDP suggest a stronger effect of the rennetting agent than was observed with hard or semi-hard cheese types (Emmental and Trappist, resp.) manufactured from cow's milk. The values of total protein as well as of water and alcohol soluble PDP content were found to be significantly different in the batches prepared in parallel with *CR* and *MR*, resp. In 3 out of 4 cases the use of *MR* brought about a higher total protein content of cheese, in one case (batches 10 and 10.1) the sense was opposite. However, the difference was slight, although significant. In all the batches obtained with *MR* alcohol soluble PDP content was considerably higher as compared to those prepared with *CR*. The respective ratios ranged from 1.5 to nearly 2.0. The differences in water soluble PDP content as found in cheeses manufactured with *CR* and *MR*, resp., are contradictory and do not permit of any conclusion.

2.1.4. White cheese. The results obtained with the soft type White cheese manufactured from ewe's milk are shown in Table 4.

Table 4

Comparison of total and soluble protein content of White cheese batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch	Year of production	Type of rennet used	Concentration of rennet added to 100 l milk, SU · 10^3	Protein content of cheese					
				total % (w/w)		water soluble		alcohol soluble	
						related to total protein, %			
				$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
11	1973	*CR*	45	15.7	0.1	10.9	1.5	9.3	1.1
11.1	1973	*MR*	30	14.6**	0.0	$12.7^{\varnothing}$	0.7	12.8*	1.7
11.2	1973	*MR*	37	13.8***	0.1	14.7*	0.5	13.0**	0.4

For symbols and explanations see Tables 1, 2 and 3.

The data indicate a distinct influence of the coagulant on total protein as well as on alcohol soluble PDP content. The former characteristic was found to be lower, the latter considerably higher in the batches manufactured with *MR*.

2.1.5. Comparison of the relative contents of soluble PDP in different cheese types. Some of the results presented in Tables 1—4 are summarized in Fig. 1.

Fig. 1 gives a clear picture on the considerable differences in the distribution of soluble PDP found between the various cheese types irrespective

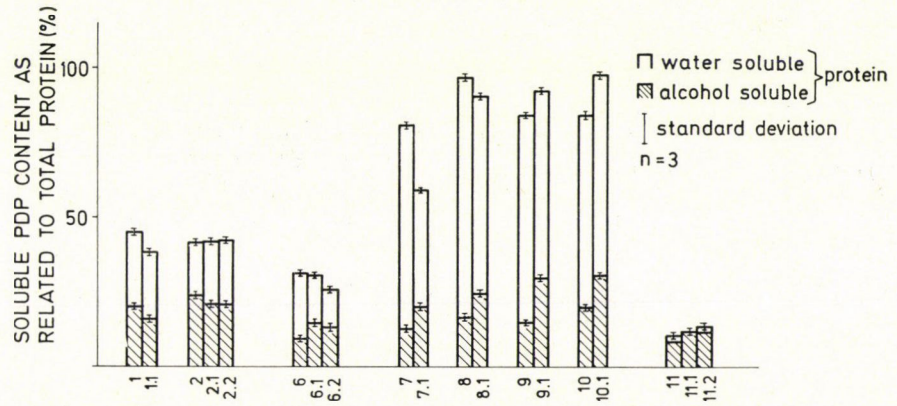

Fig. 1. Comparison of the relative contents of soluble protein degradation products (PDP) in different cheese types. 1, 2, 6, 7, 8, 9, 10, 11: cheese manufactured with calf rennet *(CR)*; 1.1, 2.1, 2.2, 6.1, 6.2, 7.1, 8.1, 9.1, 10.1, 11.1, 11.2: cheese manufactured with microbial rennet *(MR)*. Cheeses of a given type marked with identical first numerals have been manufactured in parallel with *CR* and *MR*, resp. Types of cheese, years of production (in brackets) and numbers of batches are as follows: Emmental (1972), 1, 1.1; (1973), 2, 2.1, 2.2; Trappist (1973), 6, 6.1, 6.2; "Pálpusztai" (1973), 7, 7.1, 8, 8.1; (1974), 9, 9.1, 10, 10.1; White cheese (1973), 11, 11.1, 11.2. For the determination of total protein and soluble PDP content see para. 1.2

of the type of coagulant used in the manufacturing process. In mature Emmental cheese about 40% of the total protein content have been degraded in the ripening process to soluble products, about half of which are alcohol soluble, *i.e.* consist of small peptides and free amino acids. In Trappist cheese the relative percentage of soluble PDP is somewhat lower (below 30% of total protein) and again about half of these are alcohol soluble. In "Pálpusztai"

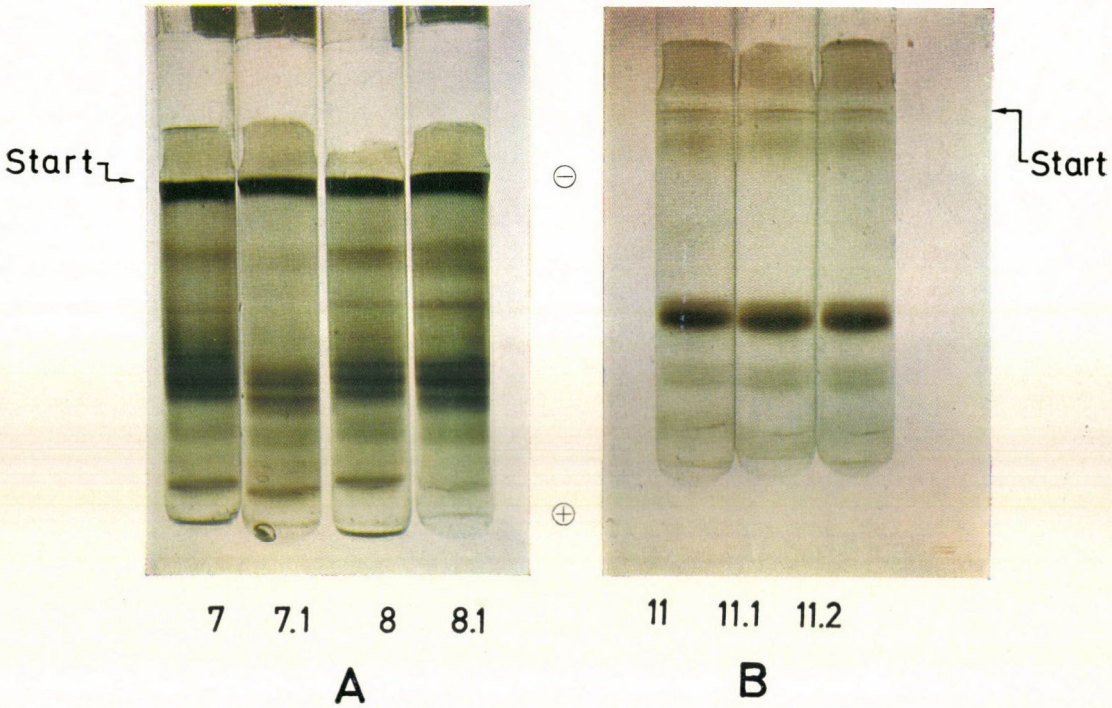

Fig. 2. Polyacrylamide gel disc electrophoretograms of the water soluble proteins of "Pálpusztai" (A) and White cheese (B). 7, 8 and 11: batches prepared with calf rennet *(CR)*; 7.1, 8.1, 11.1 and 11.2: batches prepared with microbial rennet *(MR)*. Batches marked with identical first numerals have been manufactured in parallel with *CR* and *MR*, resp. For details of electrophoresis see para. 1.3 for those of cheese characteristics Tables 3 and 4,

cheese the greater part of total protein (59 to 97%) is soluble, but only a relatively low percentage consists of low molecular weight compounds. The amount of these varies within wide limits from batch to batch and also with the type of rennet used. The soft type White cheese produced from ewe's milk presents an entirely different pattern of soluble PDP in that it contains only a low relative percentage of solubilized compounds (10 to 15%). These, however, consist nearly entirely of small peptides and free amino acids.

2.2. *Electrophoretic fractionation of soluble PDP of soft cheese*

The disc electrophoretograms of the water extracts of "Pálpusztai" and White cheese batches manufactured with *CR* and *MR*, resp., are shown in Fig. 2.

The electrophoretic patterns of the soluble proteins of the two kinds of soft cheese are rather different with respect both to the number and to the location of the fractions. Less marked differences or none whatever could be observed between the batches of a given cheese type, whether manufactured in parallel with different types of rennet or prepared with the same coagulant at different times, *i.e.* from various milk batches.

12 to 15 protein zones could be discerned by the naked eye on the electrophoretograms of "Pálpusztai" extracts and 5 on those obtained from White cheese. The relative mobilities (M_B) and estimated colour intensities of the stained protein bands are given in Tables 5 and 6.

As can be seen from the tables, only one pair of "Pálpusztai" batches (8 and 8.1) showed differences with respect to the number of protein zones. These arose from the absence of some faint bands from the soluble protein patterns of the cheese manufactured with the microbial enzyme preparation. The other pair of "Pálpusztai" yielded only minor differences owing to the alternate presence or absence of some poorly stained bands, while practically identical electrophoretograms were obtained for the water soluble proteins of all the White cheese batches investigated.

The densitograms of the "Pálpusztai" batches 8 and 8.1 as well as of the White cheese batches 11, 11.1 and 11.2 are shown in Fig. 3.

On the densitograms of the electrophoretically separated soluble protein fractions of the "Pálpusztai" batches 8 and 8.1 as manufactured with *CR* and *MR*, resp., 8 peaks each could be observed, one of the two largest having two "shoulders". The number of the peaks obtained by densitometry was considerably reduced as compared to the readings performed by the naked eye. This is obviously due to insufficient sensitivity of the instrument used. Another difficulty of instrumental evaluation consisted in the overlapping of peaks although the stained zones seemed well separated. For comparative purposes it was found, therefore, expedient to form 5 groups of the 8 peaks as indicated in the figure by vertical bars and Roman numbers. The colour intensities of these groups of fractions as calculated by the integrator of the instrument were then expressed as percentage of total colouration. The histograms thus obtained are shown in the lower part of Fig. 3. Although instrumental evaluation did not prove too sensitive, the difference in soluble protein composition of the "Pálpusztai" batches produced with different coagulants could clearly be established in that the fastest moving band did not appear in the densitogram corresponding to the cheese manufactured with *MR*.

As to the electrophoretograms of the water soluble proteins of White cheese, instrumental evaluation proved less sensitive than the naked eye in this case as well: only 4 peaks appeared on the densitogram instead of 5. These were, however, well separated and relative colour intensities could be

Table 5

Relative mobilities of electrophoretically separated water soluble protein fractions of "Pálpusztai" cheese batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch →	7		7.1		8		8.1	
No. of fractions	$M_B \cdot 100$	I	$M_B \cdot 100$	I	$M_B \cdot 100$	I	$M_B \cdot 100$	I
1	0	+++++	0	+++++	0	+++++	0	+++++
2	8	+	9	+	—		—	
3	15	+	15	+	14	+	—	
4	23	++	22	++	22	+++	23	++
5	27	++	28	++	28	++	27	++
6	36	+	37	+	39	+	—	
7	41	++	43	+	41	++	42	+++
8	50	+	50	++	51	+	52	+
9	56	++	—		58	++	—	
10	62	++	62	+++	64	+++++	62	+++++
11	66	++++	66	++++	68	++++	67	+++++
12	72	+++	71	++++	74	+++++	72	++++
13	—		76	++	78	++	78	++
14	82	+++	81	+++	83	+++	84	++
15	87	+++	86	+++	88	+++	89	+++
16	100	++++	100	++++	100	++++	100	++
Total number of fractions	15		15		15		12	

7 and 8 = batches prepared with *CR*; 7.1 and 8.1 = batches prepared with *MR*
M_B = relative electrophoretic mobility (migration distance as related to that of the bromophenol blue marker)
I = colour intensity of stained zone;
+++++ = very strong, black colouration
++++ = strong, black colouration
++ and +++ = different degrees of medium colouration
+ = faint, hardly visible
$s\%$ = 0.6–6.0

Table 6

Relative mobilities of electrophoretically separated water soluble protein fractions of White cheese batches manufactured in parallel with calf rennet (CR) and microbial rennet (MR)

Sign of batch →	11		11.1		11.2	
No. of fractions	$M_B \cdot 100$	I	$M_B \cdot 100$	I	$M_B \cdot 100$	I
1	7—12	+	8—12	+	7—12	+
2	63	+++	62	+++	61	+++
3	67	+++++	66	+++++	67	+++++
4	81	++	81	++	82	++
5	87	++	86	++	87	++
Total number of fractions	5		5		5	

11 = batch prepared with *CR*
11.1 and 11.2 = batches prepared with *MR*
M_B = relative electrophoretic mobility (migration distance as related to that of the bromophenol blue marker)
I = colour intensity of stained zone:
+++++ = very strong, black colouration
++ and +++ = different degrees of medium colouration
+ = faint, hardly visible
$s\%$ = 0.7—2.3

calculated individually for every peak. No significant difference could be established between the soluble protein patterns of the batches manufactured with different rennetting agents.

3. Conclusions

Results obtained as to total protein content and to protein degradation products were very different for the various cheese types investigated. Thus, no general conclusion as to the effect of the rennetting agent on these cheese constituents can be drawn.

Total protein content in hard and semi-hard cheese as manufactured from cow's milk (Emmental and Trappist) did not seem affected by the type of rennet used (Tables 1 and 2). The two soft cheese types investigated, "Pálpusztai" from cow's milk and White cheese from ewe's milk, showed significant differences in this respect with different types of rennet. The microbial enzyme preparation had an opposite effect on the two soft cheese types: while in most cases it increased the total protein content of "Pálpusztai", it decreased that of White cheese.

The differences in water soluble PDP content as observed between batches manufactured in parallel with *CR* and *MR*, resp., are divergent for

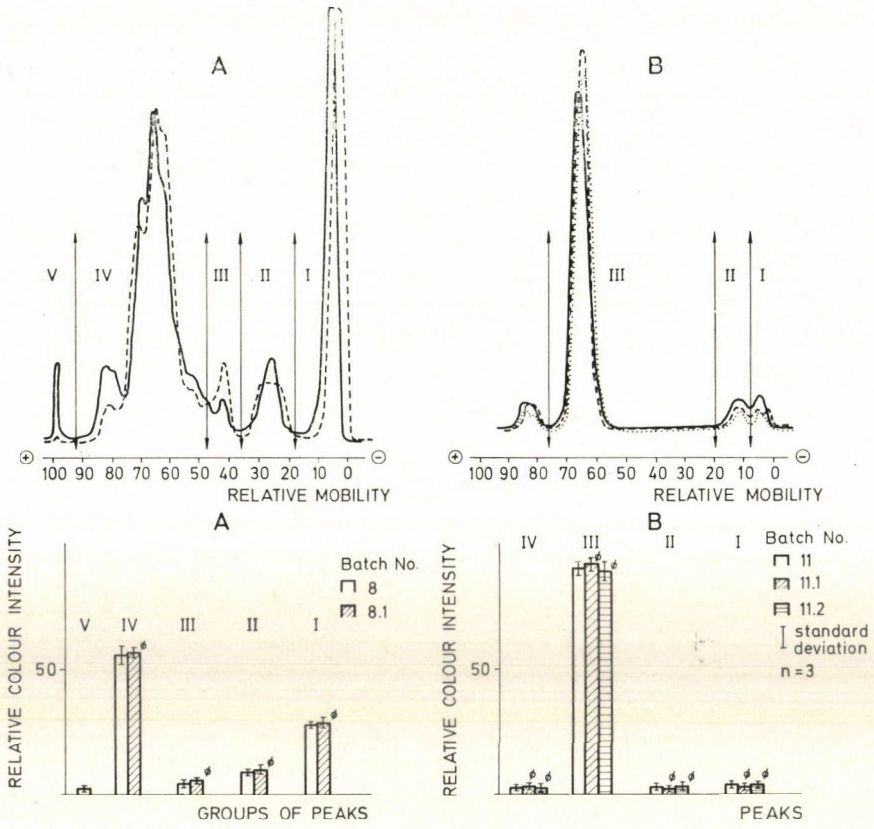

Fig. 3. Distribution of colour intensity in the electrophoretically separated and stained soluble protein fractions of "Pálpusztai" (A) and White cheese (B). — Upper part: densitometric curves corresponding to the colouration of the protein bands as obtained for the electrophoretograms 8, 8.1, 11, 11.1 and 11.2 represented in Fig. 2, by measurement with the densitometer *Chromoscan* (JOYCE-LOEBL, UK). ———— batches manufactured with calf rennet *(CR)*: 8 and 11; – – – – – batches manufactured with microbial rennet *(MR)*: 8.1 and 11.1; · · · · · · batch manufactured with *MR*: 11.2. 8 and 8.1 are batches of "Pálpusztai", 11, 11.1 and 11.2 batches of White cheese. The Roman numbers indicate the peaks and groups of peaks, resp., used to calculate relative colour intensities. — Lower part: comparison of relative colour intensities of electrophoretically separated and stained soluble protein zones calculated from areas of peaks and groups of peaks, resp., as related to total colouration (total area). For symbols see upper part of the figure. *n* stands for gel tubes from 2 separate electrophoretic runs performed with the same cheese extract. ø = difference between relative colour intensities of soluble protein fractions in cheeses manufactured with *CR* and *MR*, resp., not significant

Emmental and Trappist cheese and, therefore, difficult to interpret. From Fig. 1 it may, however, be clearly seen that the relative concentration of higher molecular weight PDP (insoluble in alcohol and calculated by subtracting the value of alcohol soluble PDP from that of water soluble PDP) undergoes but slight changes in Emmental, whereas it is distinctly decreased in Trappist by the use of *MR*.

A distinct but opposite effect on the formation of low molecular weight (alcohol soluble) PDP can be noted with above two cheese types: in Emmental batches produced with *MR* the values of alcohol soluble PDP are lower, in Trappist higher than in the control batches.

It seems that in hard and semi-hard cheese the changes in total protein and soluble PDP content as induced by the use of *MR* are not too important. This is corroborated for Emmental by BOLLIGER and SCHILT (1969) who found very slight differences between cheese prepared with calf rennet and with Sure Curd, resp., the latter being a preparation derived from *Endothia parasitica* and manufactured by PFIZER (USA). As for Trappist, results obtained earlier in this laboratory with a large number of commercial and experimental batches prepared with calf rennet, show the variations observed in the present study as attributed to the coagulant to be within the normal limits (EL-NOCKRASHY, 1971; EL-NOCKRASHY *et al.*, 1972, 1973; VÁMOS-VIGYÁZÓ *et al.*, 1975a).

The most striking differences in soluble PDP content between batches obtained with *CR* and *MR*, resp., were found in the soft type cheese "Pálpusztai" (Table 3). The use of microbial rennet seems to promote the formation of alcohol soluble, low molecular weight compounds (small peptides and free amino acids) in this cheese type. The effect of the type of coagulant applied shows also in the composition of soluble proteins as revealed by polyacrylamide gel disc electrophoresis (Figs. 2 and 3, Table 5). Quantitative evaluation of the number and relative colour intensities of the electrophoretically separated, stained soluble protein zones showed these to differ more or less in batches prepared with different coagulants (batches 7 and 7.1 or 8 and 8.1, resp.), while practically no differences could be detected between batches prepared with calf rennet at different times, *i.e.* from different milk batches. These findings are in good agreement with those reported by others (EDWARDS & KOSIKOWSKI, 1969; VANDERPOORTEN & WECKX, 1972) for Cheddar and Gouda cheese, resp.

In the case of White cheese, too, the use of *MR* had a favourable effect on the formation of low molecular weight nitrogenous compounds (Table 4). No differences were, however, obtained in the composition of soluble proteins as separated by gel electrophoresis (Figs. 2 and 3, Table 6). The uniformity of the electrophoretograms of the water extracts of cheese batches manufactured with different coagulants as well as the small number of protein bands may be connected with the fact that soluble protein of this cheese type is nearly entirely composed of low molecular weight (alcohol soluble) degradation products which — at least in part — do not stain with Amido Black.

On the whole it can be said that in all the cheese types investigated protein degradation seems to be more or less affected by the type of coagulant

used. The changes induced by the microbial rennet are particularly marked in soft type cheeses. No relationship could, however, be established between protein content and/or composition of cheese and variations in the amount of *MR* used in the manufacturing process.

*

The authors are indebted to Professor Dr. R. TARJÁN, Director and Dr. E. DWORSCHÁK, Head of Department of the INSTITUTE OF NUTRITION, Budapest for having made possible the evaluation of the electrophoretograms with the *Chromoscan* densitometer. The skilful laboratory work of Mrs. É. SÁNTHA is highly appreciated.

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Acta Alimentaria, Vol. 4 (4), pp. 405—412 (1975)

ACTIVATION ANALYSIS OF TRACE ELEMENTS IN PAPRIKA

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(Received May 12, 1975)

Various samples of Hungarian spice paprika (powdered) were analysed for their Br, Fe, Co and Mn content. Samples of paprika both with and without seeds were studied. The values are reported in ppm (dry weight).

Statistical analysis showed significant differences in the trace element contents between most cultivars. In the majority of the cases, increasing the seed content of the samples tended to reduce their trace element content.

The role of trace metals in biological systems is extremely complex. The absence of certain trace elements can be detrimental toward health, while an excess of the same elements may be toxic. Although the exact role of the elements may be somewhat unclear, the list of ions now considered essential has grown from only iron, in the 17th Century, to now include some 13 elements (SCHWARZ, 1972).

It is important, therefore, to measure the trace element content of foodstuffs in an attempt to increase one's understanding of the distribution of elements both geographically and throughout the food chain.

1. Materials and methods

The powdered paprika samples were supplied by the PAPRIKA RESEARCH STATION, Kalocsa, Hungary (Table 1).

All analyses were made by comparing the spectra to those of known purity (pro anal.) and concentration (MERCK, CHEMICAL Co.). Samples were measured with an *Intertechnique* 400-channel pulse height analyser connected to a 12 cm^3 Ge/Li detector.

Long half-life elements. — Samples weighing from 0.2—0.4 g were sealed in quartz ampules. The samples were irradiated for 24 hours in a neutron flux of $3—5 \cdot 10^{13}$ n $cm^{-2} s^{-1}$. After waiting 7 days, the samples were counted.

Short half-life elements. — Samples weighing from 0.08—0.15 g were sealed in PVC ampules and irradiated for 10 minutes in a neutron flux of $3—5 \cdot 10^{13}$ n cm^{-2} s^{-1}.

After waiting 3 hours the samples were counted.

Samples were irradiated with thermal neutrons, therefore, secondary interfering reactions did not occur.

Table 1

Paprika cultivars studied in this report

No.	Samples	
1.	**K—D—601**	determined growth type, not hot, without seeds
2.	**K—D—601**	determined growth type, not hot, with 20% seeds
3.	**K—D—621**	determined growth type, hot, without seeds
4.	**K—D—621**	determined growth type, hot, with 20% seeds
5.	**K—M—622**	half determined growth type, not hot, without seeds
6.	**K—M—622**	half determined growth type, not hot, with 20% seeds
7.	**K—504**	continuous growth type, not hot, without seeds
8.	**K—504**	continuous growth type, not hot, with 20% seeds
9.	**K—V—1**	continuous growth type, hot, without seeds
10.	**K—V—1**	continuous growth type, hot, with 20% seeds

The red pepper *(Capsicum annuum)* cultivars were grown in the vicinity of Kalocsa (K) in Hungary.

2. Results

Figure 1 is a reproduction of a typical spectrum obtained by the activation of paprika. The peaks measured were: ^{82}Br 777 keV, ^{59}Fe 1099 keV and ^{60}Co 1330 keV. The short half-life ^{56}Mn was measured at 847 keV.

The mean values and standard deviations of the samples can be found

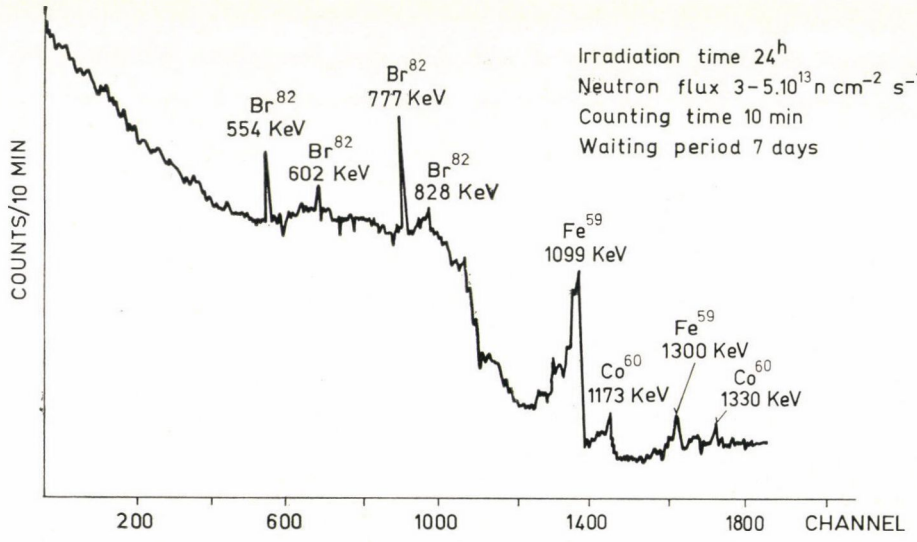

Fig. 1. Typical spectrum of neutron-activated paprika

Table 2

Average value (ppm in the dry matter) and standard deviation (n = 5) of bromine, iron, cobalt and manganese contents in powdered paprika samples

No. of sample	Br		Fe		Co		Mn	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
1	24.19	1.75	127.02	1.28	4.96	0.24	18.21	0.65
2	14.56	1.46	137.84	0.71	6.03	0.16	11.44	0.39
3	10.92	1.09	143.20	2.75	4.68	0.67	8.79	0.94
4	21.54	1.48	119.12	7.67	4.52	0.17	13.14	0.76
5	11.55	1.24	125.18	0.66	6.21	0.42	10.66	0.69
6	14.16	0.79	110.94	0.81	4.58	0.34	10.09	0.57
7	15.93	0.82	118.00	2.09	5.27	0.29	12.08	1.16
8	14.76	0.35	106.34	3.64	3.51	0.55	9.83	0.48
9	18.63	1.08	100.54	2.90	3.69	0.32	16.66	0.94
10	11.34	0.38	104.36	4.06	4.79	0.15	9.04	0.95

in Table 2 which compares the bromine, iron, cobalt and manganese contents of the examined paprika samples ground with or without seeds (Figs. 2—5) using the *Student t* test.

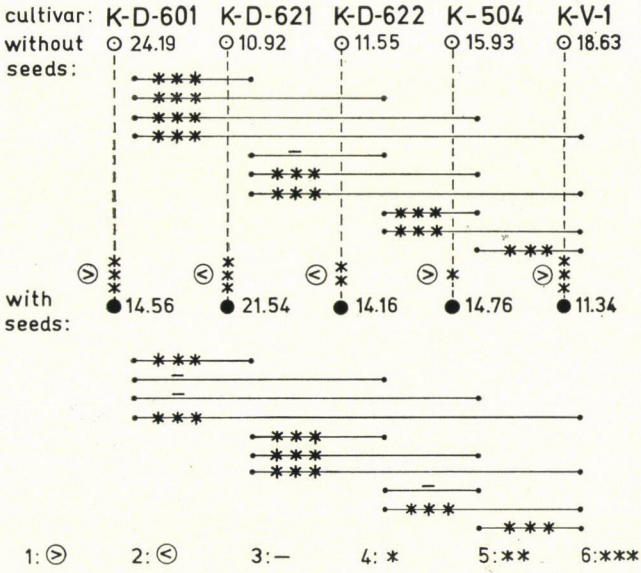

Fig. 2. Significance of differences in the bromine contents of powdered paprika samples, calculated by the *t* test (Figure: average values in ppm in the dry matter). 1: Br content of sample without seeds higher than of that with seeds; 2: Br content of sample without seeds lower than of that with seeds; 3: difference not significant; 4: difference significant ($P \geq 95\%$); 5: difference highly significant ($P \geq 99\%$); 6: difference very highly significant ($P \geq 99.9\%$)

As can be seen, the iron content is much higher than that of the other trace metals. It is not however, inordinately high for plant samples. CHATFIELD and ADAMS (1940) and KAPELLER (1971) for example, have measured the iron content of many vegetables and found it to vary from 6.6 to 76 ppm

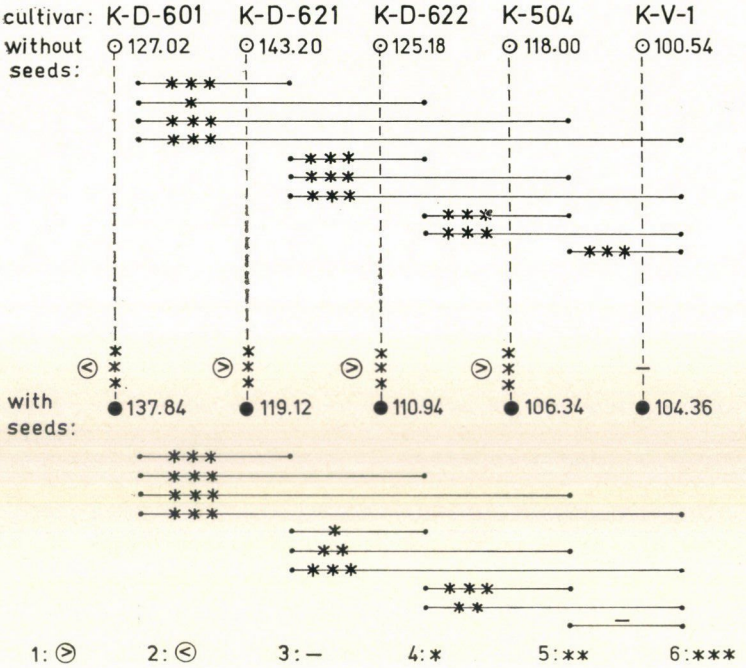

Fig. 3. Significance of differences in the iron contents of powdered paprika samples: calculated by the t test (Figure: average values in ppm in the dry matter). 1: Fe content of sample without seeds higher than of that with seeds; 2: Fe content of sample without seeds lower than of that with seeds; 3: difference not significant; 4: difference significant ($P \geq 95\%$); 5: difference highly significant ($P \geq 99\%$); 6: difference very highly significant ($P \geq 99.9\%$)

wet weight. MAYER and GORHAM (1951) and CANNON (1960) have also reported that normal iron content may be as high as 160 ppm.

There are even some measurements on algae which indicate iron contents as large as 10 000 ppm (DRASKOVIC *et al.*, 1972). The same is true for the other elements measured. Co may vary from 0.5 ppm in a non-accumulator to as high as 1.8% dry weight in an accumulator species such as *Crotolaria cobalticola* (PETERSON, 1972). Br and Mn also vary greatly depending upon the species of plant investigated (BOWEN, 1967; UNDERWOOD, 1962).

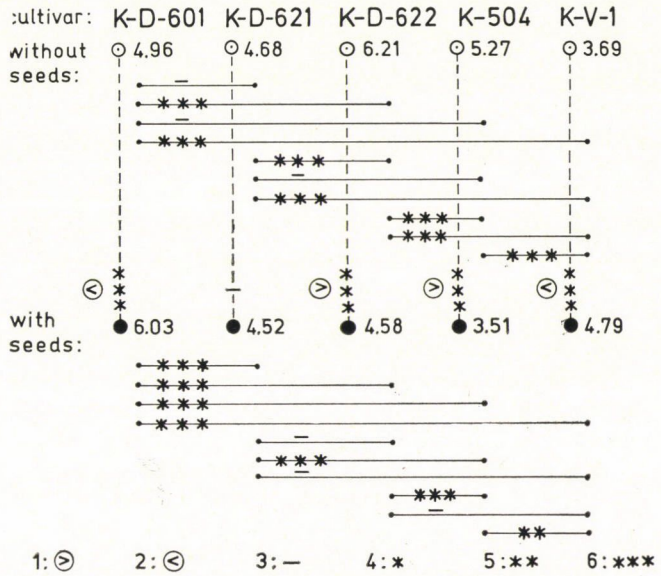

Fig. 4. Significance of differences in the cobalt contents of powdered paprika samples, calculated by the t test (Figure: average values in ppm in the dry matter). 1: Co content of sample without seeds higher than of that with seeds; 2: Co content of sample without seeds lower than of that with seeds; 3: difference not significant; 4: difference significant ($P \geq 95\%$); 5: difference highly significant ($P \geq 99\%$); 6: difference very highly significant ($P \geq 99.9\%$)

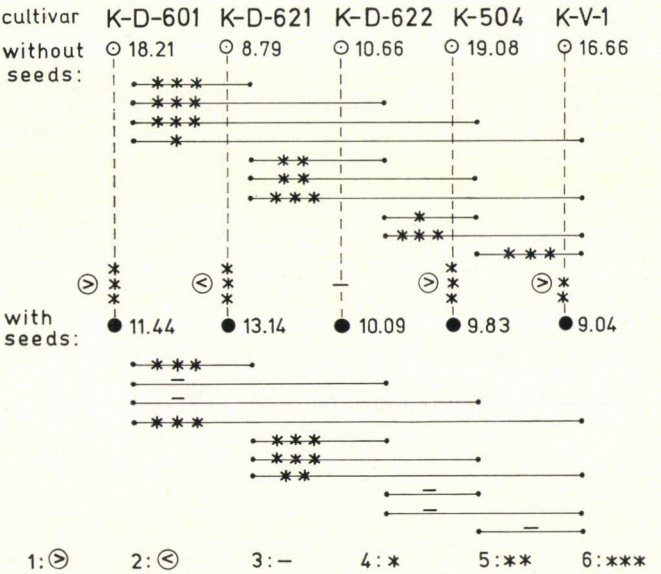

Fig. 5. Significance of differences in the manganese contents of powdered paprika samples calculated by the t test (Figure: average values in ppm in the dry matter). 1: Mn content of sample without seeds higher than of that with seeds; 2: Mn content of sample without seeds lower than of that with seeds; 3: difference not significant; 4: difference significant ($P \geq 95\%$); 5: difference highly significant ($P \geq 99\%$); 6: difference very highly significant ($P \geq 99.9\%$)

Table 3

Effect of increased seed content of ground paprika (5 cultivars) on the changes in their contents of 4 trace elements

Trace element		Cultivar					No. of cultivars in the "higher", "lower" or "not differing" categories			Total
		K–D–601	K–D–621	K–D–622	K–504	K–A–1	>	<	—	
Br		>	<	<	>	>	3	2	0	5
Fe		<	>	>	>	—	3	1	1	5
Co		<	—	>	>	<	2	2	1	5
Mn		>	<	—	>	>	3	1	1	5
No. of trace elements in the "higher",	>	2	1	2	4	2	11			
"lower" or	<	2	2	1	0	1		6		
"not differing" categories	—	0	1	1	0	1			3	
Total		4	4	4	4	4				20

>: indicates higher trace element content in samples without seeds
<: indicates lower trace element content in samples without seeds
—: indicates no statistically significant difference

3. Conclusions

In the related pairs of samples (identical variety, ground with or without seeds) and also in all samples disregarding a few exceptions — there is a significant difference as to the amounts of the elements studied.

The significance of differences is of various extent. (The greater amount is to be found in sample ground either with or without seeds.) It cannot be concluded definitely whether the enrichment of the elements occurs in the pod tissue of the fruit or in the seeds, although, in the majority of the cases, the trace element content of the samples without seeds appeared to be significantly higher than that of the samples containing 20% seeds. This was true for all four elements with **K—504**, for two elements (Br and Mn) with cultivars **K—D—601** and **K—V—1** and for two other elements (Fe and Co) with **K—D—622**. With cultivar **K—D—621**, such a situation was found to apply only to one element (Fe) (Table 3).

Viewed differently, the trace element content of samples not containing seeds was higher than that of those with seeds in 3 of the 5 cultivars examined for 3 elements (Br, Fe, Mn) and in 2 cultivars for 1 element (Co).

The present examinations are rather of exploratory nature and they were carried out in the first place with the aim at testing the applicability of the method in food analysis. However, it would be useful to extend the experiments from the point of view of plant and human physiology. In order to get a closer view of the background it is necessary to examine the soil, agrotechnics, plant physiology, yield, as well as the industrial processing of paprika, accumulation of the elements in the human organism.

*

The authors are indebted to the Austrian BUNDESMINISTERIUM FÜR GESUNDHEIT UND UMWELTSCHUTZ for financial support. The authors are indebted to Dr. K. KAPELLER, INSTITUTE FOR VEGETABLE GROWING, PAPRIKA RESEARCH STATION, Kalocsa and Mrs. M. HARKAY-VINKLER, RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRIES, Budapest for the supply of experimental paprika samples.

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Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója. Műszaki szerkesztő: Botyánszky Pál
A kézirat nyomdába érkezett: 1975. VIII. 6. — Terjedelem: 7,25 (A/5) ív 30 ábra, (1 színes)

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27. II. 1976